LIVER DAMAGE IN ACUTE HELIOTRINE POISONING

1. STRUCTURAL CHANGES

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Many plants of the genera Senecio and Erectites, of the family Compositae, Crotalaria, a genus of Leguminosae, and Heliotropium and two other genera of Boraginaceae all contain alkaloids capable of selectively damaging the liver of domestic animals. These alkaloids are similar in chemical structure, being esters of a basic alcohol possessing a pyrrolizidine ring.

In Australia natural outbreaks of liver disease in sheep, horses and cattle have occurred due to *Heliotropium europaeum, Crotalaria retusa, Senecio cunninghamii* and *S. jacobaea*. In Victoria, South Australia and the Riverina, the outbreaks usually involve consumption of *H. europaeum* (potato weed) by sheep.

References to the early work in which the association was suspected are available in the report of Bull, Dick, Keast and Edgar (1956). They established the natural history, pathology, and symptomatology of the outbreaks by conducting a systematic investigation of chronic poisoning in sheep due to consumption of the summer annual *H. europaeum*. They showed that it produced selective liver injury of a peculiar type, which, when established, could progress spontaneously to liver failure.

Bull (1955) had reviewed the histological changes in the liver in chronic heliotrope poisoning of sheep and Crotalaria poisoning of horses. He emphasized that gross enlargement of the individual liver cells, "megalocytosis", was a constant and characteristic feature of both.

These facts suggested that experimental use of the active principles might provide pathological information of general application in the understanding of liver disease. Since the recent chemical work by Culvenor, Drummond and Price (1954), and by Culvenor (1954), five alkaloids—inclusive of heliotrine and lasiocarpine (Trautner and Neufeld, 1949)—and their five *N*-oxides, have now been isolated from *H. europaeum*, and have thus become available in pure form for biological study.

Harris, Rose and Chen (1957) have reported a short pharmacological study using heliotrine in rats.

Schoental and Magee (1957) described the chronic liver changes which may follow the administration of a single dose of lasiocarpine to rats.

Bull, Dick and McKenzie (1958) have summarized the acute toxic effects of heliotrine and lasiocarpine and their *N*-oxides on the rat, but only brief references to the liver changes due to heliotrine was possible in the compass of this informative paper.

The present paper records histological changes observed in a large series of rat livers during the course of acute poisoning by a high dose of the pure alkaloid heliotrine. As the same livers were used for biochemical studies, it has been possible to correlate the information derived from each method of investigation.

Methods.

Eighty-one adult male Wistar rats, weighing between 240 and 340 gms. were used, including 22 controls. Heliotrine was administered in aqueous solution neutralized to pH 7·3 with HCl, at a concentration of 320 mg. in 25 ml. The dose used was 320 mg./kg. body weight, injected intraperitoneally, under brief ether anaesthesia. Animals were killed by stunning and exsanguination at various times after the injection, and the livers rapidly removed, weighed and transferred to ice-cold 0·25 M. sucrose. Samples were taken for biochemical study and the remainder sliced and fixed in formalin buffered to neutral pH with sodium acetate.

Paraffin sections were cut at 5μ. Masson's method and haematoxylin and eosin were used for general staining, Mallory's phosphotungstic acid haematoxylin was used for mitochondria, and Weigert's fibrin stain and the periodic acid Schiff method for inclusion globules.

Frozen sections were cut at 5μ by Louis's technique (1957) and were stained for lipid with Fat red (Chroma) and Sudan black (each in 60 p.c. alcohol) and with 1 p.c. aqueous Nile blue sulphate.

Samples of the fatty droplets were prepared from 10 p.c. homogenates of fresh liver tissue in 0·25 M. sucrose by centrifugation at ten thousand *g*. The yellow fatty layer collected at the upper end of the tube, and was separated and washed twice by resuspension in 0·25 M. sucrose and subsequent centrifugation. Microscopic examination by phase contrast showed that the preparation was free from normal mitochondria.

Succinoxidase activity was measured by the method of Schneider and Potter (1943).

Nucleic acid was estimated by the method of Schmidt and Thannhauser (1945).

RESULTS.

Macroscopic findings. During the first 12 hours a progressive swelling, pallor and friability of the liver developed. Between the 12th and 16th hours the liver became mottled with dark red spots which were uniformly distributed throughout the substance, apparently with a lobular distribution. After the 20th hour the whole liver rapidly became swollen, tense, friable, and deep purple in colour, and remained in this state until death of the animal.

Microscopic findings. Under the experimental conditions it was found that the severity of the injury at a given time after administration of heliotrine showed satisfactory consistency from animal to animal. The evolution of the major changes may be seen in the low-power photomicrographs (Figs. 1 to 4). As early as the first hour there was a reduction of cytoplasmic basophilia of the inner half of the lobules and occasional cells showed more severe changes. At 4 hours (Fig. 1) the inner 2/3 of the lobules showed cytoplasmic swelling and

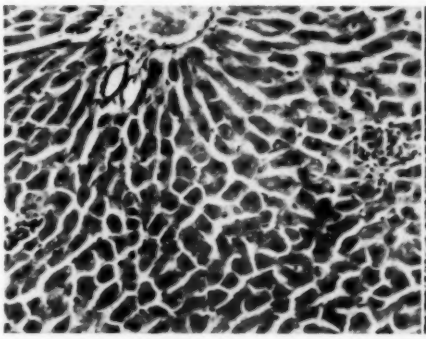

Fig. 1. Photomicrograph of the liver of a rat poisoned 4 hours previously by heliotrine, showing two patches of necrosis in the middle zone and dilatation of a lymphatic vessel in the portal canal. (× 150)

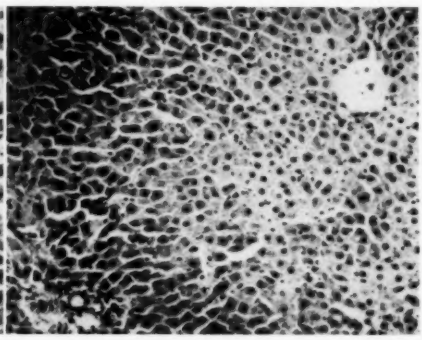

Fig. 2. Photomicrograph showing swelling and pallor of the cells of the central and middle zones of the liver lobules, 16 hours after heliotrine administration. Patches of necrosis are also present. (× 100)

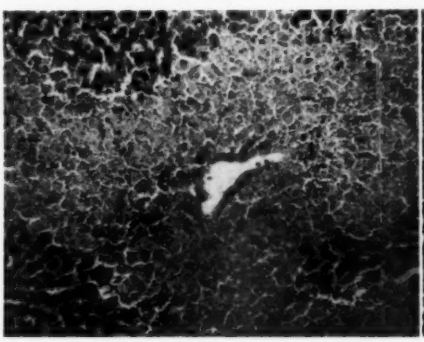

Fig. 3. Photomicrograph, 24 hours after heliotrine poisoning, showing necrosis of the middle and central zones. A few cells around the central vein have survived. Haemorrhage has already taken place into the necrotic tissue. (× 100)

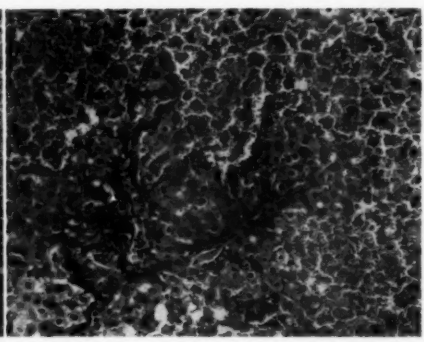

Fig. 4. Photomicrograph, 28 hours after heliotrine administration. Even the surviving cells of the portal zone are swollen. The necrotic cells of the middle and central zones are shrinking and are in contact with red blood cells. (× 100)

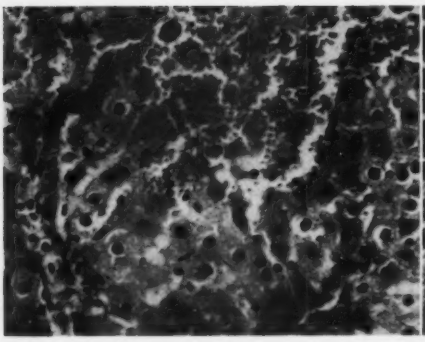

Fig. 5. Photomicrograph of the central part of the field shown in Fig. 4. The cytoplasm of the surviving cells is pale, swollen and vacuolated by small fatty droplets. Red blood cells are packed around the necrotic cells. (× 200)

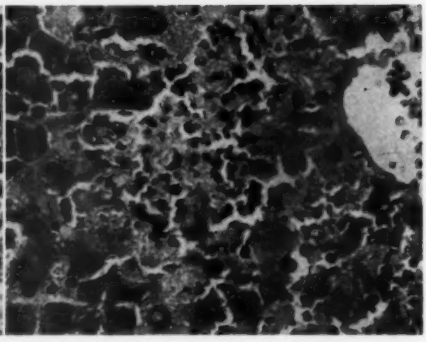

Fig. 6. Photomicrograph, 20 hours after heliotrine administration, showing degenerative cytoplasmic and nuclear changes and early haemorrhage. The maximum localization is in the middle zone, a few cells around the central vein still survive. (× 200)

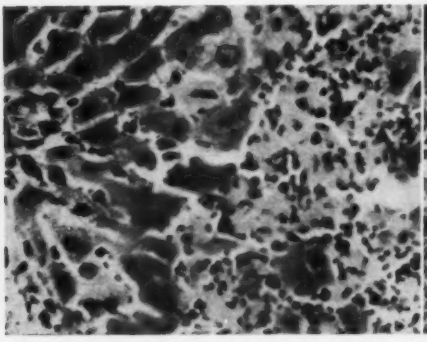

Fig. 7. Photomicrograph, 20 hours after heliotrine administration, showing various types of degenerative nuclear change. (× 200)

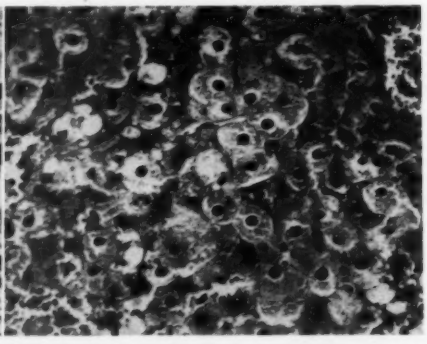

Fig. 8. Photomicrograph, 28 hours after heliotrine poisoning. Hydropic change has reached the portal canal (top right). (× 200)

fine frothy vacuolation, and patches of necrosis involving small clumps of cells were present in the mid-zones. The lymphatics of the portal canals were distended.

Over the next 10 hours the cytoplasmic changes became progressively more severe and the patchy necroses more numerous. By the 16th hour all cells showed some degree of damage and patches of necrosis were present in every lobule, involving single cells, and small and large groups of cells. Small necroses

were so common that the trabeculae were indistinct and fragmented. At this stage all the cells showed advanced swelling and frothy change of the cytoplasm, and in some places these processes had produced typical hydropic change (Fig. 8).

After 16 hours the patches of necrosis became confluent, especially in the mid-zones, to form large islands, and pooling of red cells was taking place in the necrotic areas.

By the 20th hour (Fig. 3) necrosis was confluent in the middle and inner zones although a rim of surviving cells was often present immediately around the central veins. Each portal canal was surrounded by a collar of surviving cells, although these showed serious cytoplasmic swelling, frothy vacuolation and considerable nuclear swelling.

Between the 24th hour and the 29th hour (Fig. 4) slow extension of necrosis into the periportal zone took place. Haemorrhage into the necrotic tissue was now very gross (Figs. 4 and 5) and shrinkage and crumbling of the cells was occurring.

Death often occurred between 30 and 40 hours although some animals survived for 5 days. The liver then showed lobular disorganization, gross nuclear swelling and a remarkable absence of signs of regeneration.

Some of the cytological changes are illustrated in the high power photomicrographs (Figs. 5 to 8). The major cytoplasmic changes prior to necrosis were swelling, apparently due largely to water imbibition, and frothy vacuolation produced by numerous minute droplets, ½ to 2μ in diameter.

These droplets could be resolved individually in thin frozen sections and were found to stain selectively with Sudan black and Fat red. As with neutral lipid, the droplets stained pink with 1 p.c. Nile blue sulphate. The isolated washed samples showed the same staining properties, and on analysis were found to possess succinoxidase activity, and to contain nitrogen and ribonucleic acid.

Succinoxidase is an enzyme of high activity, and is capable of surviving such adverse conditions as cell disruption and extraction by relatively crude biochemical methods. It has a predominantly mitochondrial localization in the cell. The evidence thus indicated that the minute droplets which appeared in the liver cells in heliotrine poisoning originated by infiltration of damaged mitochondria with neutral fat, as in the case of carbon tetrachloride poisoning (Christie and Judah, 1954). In heliotrine poisoning, fine droplet fatty change, although generalized, did not become so gross as in carbon tetrachloride poisoning.

The nuclei showed alterations of size and staining character. Swelling developed early and soon became generalized. It was sometimes extreme and intranuclear vacuoles then appeared. In severely damaged cells the phase of

swelling was followed by a slow shrinkage with crumpling and collapse of the nuclear membrane.

The badly damaged nuclei often showed decreased staining density leading to karyolysis, or increased density leading to pyknosis, but usually both processes occurred in the one nucleus leading to the formation of a group of small granules, an appearance commonly spoken of as "fragmentation". However, even at this stage phase contrast examination showed that the crenated and collapsed nuclear membrane was still present, surrounding the cluster of stained granules. At this time the nuclear remnant often took up a peripheral situation in the cytoplasm and might even be extruded.

Ovoid structureless hyaline vacuoles from 1 to 10μ in diameter were often present in the cytoplasm as in chronic poisoning by pyrrolizidine alkaloids (Bull, 1955). Their appearance coincided with other recognizable evidence of cell injury such as swelling and fatty change, and in the central zones they could be found from the fourth hour. Several globules of varying size were often present in a single cell, and although they were often situated near the nucleus there was no evidence that they were derived from nuclei. They could be stained with Mallory's phosphotungstic acid haematoxylin, Weigert's fibrin stain, and by the periodic acid Schiff method.

Necrosis of the cytoplasm was of the coagulative type, but was rapidly followed by shrinkage so that the cells became separated from each other leaving spaces permeated by large numbers of red cells. As the sinusoids in the necrotic areas were also disrupted, the red cells lay in direct contact with, and became adherent to, the necrotic cytoplasm (Figs. 3 to 5). There was no evidence of cessation of blood flow, or of fibrin deposition in these spaces, and fragments of disorganized cells seemed to be breaking away, to be carried into the veins. The maintenance of a circulation accounted for the absence of a static phase, in which the necrotic cells long remained unchanged, as commonly occurs in infarcts.

DISCUSSION.

It was apparent that the pyrrolizidine alkaloid, heliotrine, when administered as a massive single dose, was capable of producing recognizable signs of injury in almost every cell in as little as one hour. The uniformity and extent of the lesion throughout the liver, and its consistency from animal to animal, supported the conclusion that biochemical methods could legitimately be applied. Furthermore, histological control was valuable in interpreting the results of the biochemical studies.

Even with the large dose of alkaloid used, its action remained highly selective for liver tissue.

The early changes, consisting of loss of mitochondrial staining, cytoplasmic swelling, and fine droplet fatty change were of a histologically non-specific

character. The swelling was partly due to an increased cellular water load (Gupta, 1956) and partly due to the development of the small fat droplets from damaged mitochondria.

Haemorrhage into the necrotic areas due to sinusoidal rupture occurs only to a minor degree in acute poisoning by large doses of such hepatotoxic agents as carbon tetrachloride, dimethylnitrosamine and thioacetamide; and in general, the reticulin framework is well preserved. In the case of the pyrrolizidine alkaloids, sinusoidal rupture occurs earlier and more extensively. Selzer, Parker and Sapeika (1951) advanced evidence that retrorsine has a general action on endothelium in rats. Bull *et al.* (1958) found "blood lake" formation in the liver to be a conspicuous feature of lasiocarpine poisoning.

The Schiff positive inclusion globules which also stained with Mallory's phosphotungstic acid haematoxylin and Weigert's fibrin stain resembled those described by Bull (1955) in chronic poisoning by Senecio and Crotalaria. Their development is not an indication of chronicity, although it appears that their numbers increase with time. Their nature has not been elucidated, but the appearances suggested a degeneration product of the cytoplasm.

In acute heliotrine poisoning the swelling of the cells is due to a moderate grade of "hydropic" change caused by an increased fat and fluid content. The cytoplasmic hypertrophy, "megalocytosis" described by Bull (1955) in chronic poisoning by pyrrolizidine alkaloids, was not observed in the acute experiments.

SUMMARY.

The histological changes in rat liver after administration of the purified pyrrolizidine alkaloid heliotrine have been investigated.

With the dose used patches of coagulative necrosis were evident from the first hour. They increased in size and numbers, and by the 20th hour the middle and inner zones of the lobules were necrotic. By the 24th hour only a small rim of periportal cells survived.

Prior to necrosis the cytoplasm showed early loss of mitochondrial staining, swelling, fine fatty vacuolation, and inclusion globule formation. Destruction of sinusoids caused haemorrhage into the necrotic areas and was responsible for the intensely engorged macroscopic appearance.

Fatty change in the form of fine droplets was found to be due to infiltration of damaged mitochondria by neutral fat.

Regeneration and "megalocytosis" were not observed.

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REFERENCES.

Bull, L. B. (1955): Austral. vet. J., 31, p. 33.
Bull, L. B., Dick, A. T., Keast, J. C., and Edgar, G. (1956): Austral. J. agric. Res., 7, p. 281.
Bull, L. B., Dick, A. T., and McKenzie, J. S. (1958): J. Path. Bact., 75, p. 17.
Christie, G. S., and Judah, J. D. (1954): Proc. roy. Soc. B., 142, p. 241.
Culvenor, C. C. J. (1954): Austral. J. Chem., 7, p. 287.
Culvenor, C. C. J., Drummond, L. J., and Price, J. R. (1954): *Ibid.*, 7, p. 277.
Gupta, D. N. (1956): J. Path. Bact., 72, p. 183.
Harris, P. N., Rose, C. L., and Chen, K. K. (1957): Arch. Path., 64, p. 152.
Louis, C. J. (1957): Stain Tech., 32, p. 279.
Schmidt, G., and Thannhauser, S. J. (1945): J. biol. Chem., 161, p. 83.
Schneider, W. C., and Potter, V. R. (1943): *Ibid.*, 149, p. 217.
Schoental, R., and Magee, P. N. (1957): J. Path. Bact., 74, p. 305.
Selzer, G., Parker, R. G. F., and Sapeika, N. (1951): Brit. J. exp. Path., 32, p. 14.
Trautner, E. M., and Neufeld, O. E. (1949): Austral. J. Sci., 11, p. 211.

LIVER DAMAGE IN ACUTE HELIOTRINE POISONING

2. BIOCHEMICAL CHANGES

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In view of the peculiarly selective and severe toxic effects of the pyrrolizidine alkaloids on the liver referred to in the preceding paper (1. Histological Changes) advantage was taken of the availability of heliotrine in pure form to observe some of the pathological changes in the activities of the cell enzymes.

The respiratory mechanisms were first investigated because, in a rapid process terminating in necrosis, it seemed probable that these must eventually become involved.

METHODS.

Animals. These were the same as described in the preceding paper. Heliotrine was administered by intraperitoneal injection of an aqueous solution pH 7·3 at a dosage level of 320 mg./kg. body wt. Male rats were used throughout.

Enzyme preparations. Homogenates of rat liver were made at 0° C. in an all-glass power-driven homogenizer, with 0·25 M. sucrose as the suspending medium.

Differential centrifugation was carried out by the method of Schneider (1948).

Reagents. Cytochrome *c* was prepared by the method of Keilin and Hartree (1937) and dialyzed against distilled water.

Inorganic reagents were of analytical grade; glass-distilled water was used for all solutions, and for final rinsing of all glassware.

Diphosphopyridine nucleotide (DPN), hexokinase, and adenosine-5′-phosphate (AMP) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.; sodium pyruvate, L-malic acid, β-hydroxybutyric acid and choline chloride were obtained from L. Light and Co., Colnbrook, Bucks., England. Sodium citrate AR, sodium succinate LR and octanoic acid LR were B.D.H. reagents. Octanoic acid was purified by redistillation.

Estimations. Manometric estimations of aerobic oxidations were carried out in a standard medium in which the final concentrations in the Warburg flasks were: adenosine-5′-phosphate 0·001 M.; KCl 0·025 M.; $MgSO_4$ 0·0066 M.; cytochrome *c* $2{\cdot}7 \times 10^{-5}$ M.; sodium phosphate buffer pH 7·3, 0·16 M. Substrates were added to a final concentration of 0·01 M. in the flask volume of 2 ml. The gas phase was air, and the temperature 37° C.

Succinoxidase activity was estimated by the method of Schneider and Potter (1943). Choline oxidase activity was estimated in a medium containing sodium phosphate buffer pH 7·3, 0·04 M.; $CaCl_2$ 0·0004 M.; cytochrome *c* $2{\cdot}7 \times 10^{-5}$ M.; and choline chloride 0·05 M.

Anaerobic glycolysis was estimated under the conditions advised by Le Page (1948).

Octanoate and pyruvate oxidations were primed by 0·001 *M.* malate, the activity of which was tested in separate flasks and subtracted from the total oxygen uptake in all results quoted.

Ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) were estimated by the method of Schmidt and Thannhauser (1945).

RESULTS.

Changes in liver weight during the experimental period. The changes in liver weight during the first 30 hours are shown graphically in Fig. 1.

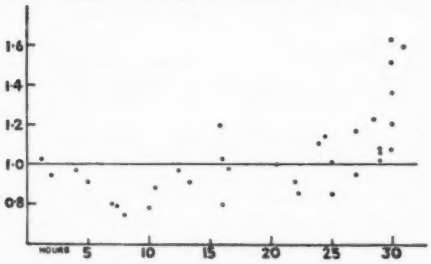

Fig. 1. Changes in liver weight during the first 30 hours of acute heliotrine poisoning. The liver weight per 100 gm. body weight of the poisoned animal is expressed as a percentage of that of the litter mate control killed at the same time.

The data were derived from 58 heliotrine-poisoned animals and 24 controls in which special care was taken to remove all lobes without injury. The liver weight per 100 gm. of body weight was then determined both for the toxic animals and for the control animal killed at the same time. The two were expressed as a ratio in which the control figure was arbitrarily regarded as unity, and thus 34 points appear on the curve. The actual figure for liver weight per 100 gm. of body weight varied considerably even with the controls – the limits being 2·44 gm. and 4·2 gm. – although the animals were all males of the same strain, on the same diet, and weighing between 250 gm. and 350 gm.

DNA and RNA levels as reference standard for enzyme activity. To enable quantitative comparisons of enzyme activity to be made with different animals over the 30-hour period, a standard of reference was necessary. In work on carbon tetrachloride poisoning, Christie and Judah (1954) were able to use DNA levels for this purpose because histological evidence indicated that cytoplasmic change was in advance of nuclear change.

In the case of heliotrine poisoning, nuclear changes appeared relatively earlier (1. Histological Changes) and analyses for DNA showed that after 15 hours the quantity per gram wet-weight had begun to fall and by 30 hours was almost half the normal (Table 1).

It was apparent that reduction of the capacity of a sample of liver to carry

TABLE 1.

Changes in Rat Liver DNA and RNA in Heliotrine Poisoning. Determinations were made in duplicate, on a pooled liver sample obtained from 2 animals. Aliquots equivalent to 1 gm. wet wt.

Animal	μg.DNA-P	μg.RNA-P
Control	280	816
Control	230	796
Control	242	804
Control	232	890
Control	250	886
Control	240	945
10 hr. heliotrine poisoned	232	790
15 hr. heliotrine poisoned	196	630
20 hr. heliotrine poisoned	169	373
30 hr. heliotrine poisoned	150	402

out a particular enzymatic activity after heliotrine poisoning might be masked if the DNA content of the sample had also fallen.

It was therefore decided to calculate enzyme activity on the basis of total oxygen uptake (or carbon dioxide output) for the quantity of liver present in the animal per gram of body weight over a given time. The gas volume used in the calculation was that actually recorded manometrically over the first half-hour of the incubation immediately following the equilibration period. Thus:

$$\text{Activity} = \frac{\mu\text{l.} \times \text{L}}{\text{E} \times \text{B}}$$

where μl. = oxygen uptake, or carbon dioxide output over the half-hour period.

L = total liver weight (gm.).

E = wet weight of liver (gm.) corresponding to the quantity of the enzyme preparation added to flasks.

B = total body weight (gm.).

Tables 2, 5 and 6 show that satisfactory agreement between experiments carried out at different times was obtained both with control and heliotrine-poisoned animals.

Homogenate respiration. The severe cytoplasmic changes observed histologically suggested that study of tissue respiration might be of value. The results of homogenate experiments are set out in Table 2 and Fig. 2 is a diagrammatic summary.

It will be observed that a major inhibition and disorganization of respiratory enzyme activity took place. The capacity to use the various substrates tested fell at different times and three major patterns were evident.

(a) Early group: The earliest respiratory change observed was reduction of pyruvate and octanoate oxidation during the first 14 hours, when the other oxidations tested were still taking place at control rates. The decline of pyruvate oxidation was gradual, and in spite of the early onset, was not complete until the 20th

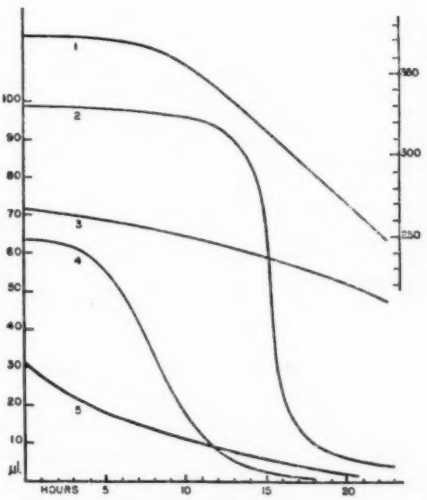

Fig. 2. Effect of heliotrine poisoning on oxidation of several substrates by rat liver homogenates. Data from Table 2. Loss of capacity to oxidize the substrates occurred in the following order:

Early—pyruvate (curve 5) and octanoate (curve 4).

Intermediate—citrate, α-ketoglutarate, glutamate, malate, β-hydroxybutyrate (curve 2).

Late—choline (curve 3) and succinate (curve 1).

The scale on the right refers to succinoxidase activity only.

TABLE 2.

Effect of heliotrine poisoning on oxidation of several substrates by rat liver homogenates. System as described in methods. Oxygen uptakes (μl.) by liver tissue/gm. body weight/½ hr.

Animal	Pyruvate	Octanoate	Citrate	Glutamate	α-ketoglutarate	β-hydroxy butyrate	Malate	Succinate	Choline
Control	39	60	80	96	108	62	85	341	83
Control	33	55	85	99	110	67	84	318	65
Control	31	58	97	101	101	62	86	366	73
Control	31	64	87	103	102	61	81	338	71
Control	26	66	95	98	101	63	—	352	69
1½ hours heliotrine	10	53	94	91	88	74	95	382	73
2 hours heliotrine	16	31	97	92	90	73	88	350	70
4 hours heliotrine	20	63	107	—	110	75	—	400	84
5 hours heliotrine	17	55	110	91	85	68	85	360	72
7 hours heliotrine	34	44	94	90	92	66	85	344	63
8 hours heliotrine	13	33	92	78	79	58	83	317	58
12 hours heliotrine	12	8	101	98	92	66	93	363	68
16 hours heliotrine	8	4	20	95	81	55	71	325	60
16 hours heliotrine	Nil	Nil	15	14	17	10	9	350	57
20½ hours heliotrine	2	19	4	6	8	6	Nil	271	40
24 hours heliotrine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	272	42
24½ hours heliotrine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	250	43
29 hours heliotrine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	218	27
29 hours heliotrine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	—	—

hour. In contrast, octanoate oxidation fell suddenly to a negligible level between the 8th and 14th hours.

(b) Intermediate group: This comprised the enzymes responsible for the oxidations of citrate, α-ketoglutarate, malate, glutamate and β-hydroxybutyrate. The activity of this group dropped from control levels to complete failure between the 14th and 20th hours; although they were still capable of considerable activity for several hours longer if DPN were added to the incubation medium.

However, even normal homogenates show a greatly increased oxygen uptake if 1 μmole of DPN is added to the flasks, although this is not necessarily due to increased oxidation of the substrate under test.

The possibility that the failure of activity of this group of DPN dependent enzymes might be due to DPN lack was therefore investigated further using freshly isolated mitochondria, in which DPN addition produces only slight increase of oxygen uptake.

(c) Late group: This comprised succinoxidase and choline dehydrogenase. The activities of these enzymes remained at control levels during the first 16 hours, and thereafter declined gradually. Even as late as 28 hours after the toxic dose, their activities were still almost two-thirds of the normal.

Anaerobic glycolysis. In experiments to test the effects of heliotrine poisoning on anaerobic glycolysis the results shown in Table 3 were obtained.

TABLE 3.

Anaerobic glycolysis of Control and Heliotrine-poisoned Rat Liver Homogenates.
Activities are stated as CO_2 production (μl.) by liver tissue/gm. body weight/¼ hr.

Control	7¼ hour heliotrine-poisoned rat	21¾ hour heliotrine-poisoned rat
113 121 118	122	130

At 21¾ hours after heliotrine administration, when aerobic oxidation was completely disorganized and most of the liver tissue histologically necrotic, anaerobic activity was undiminished.

The effect of copper ions. The livers of sheep suffering from chronic poisoning by pyrrolizidine alkaloids are known to accumulate excess copper. It therefore seemed of interest to determine whether the copper ion exerted any selective effect on the activity of the mitochondrial enzymes of the heliotrine-poisoned animal.

Glutamate was used as the test substrate, and experiments were carried out with homogenates from the control and 16-hour heliotrine-poisoned animals, after the addition of varying concentrations of copper to the incubation medium. Table 4 showed that 10^{-4} M. copper reduced the respiration of the control homogenate by 42 p.c. and of the heliotrine-poisoned homogenate by 47 p.c. This difference is not significant.

TABLE 4.

Effect of Cu^{++} on respiration of control and heliotrine-poisoned rat liver homogenates. System as described in methods. Substrate glutamate. Oxygen uptakes (μl.) by liver tissue/gm. body wt./¼ hr.

Control				16 hour heliotrine			
No Cu^{++}	Cu^{++} $3{\cdot}3\times10^{-4}$ M	Cu^{++} 10^{-4} M	Cu^{++} 10^{-5} M	No Cu	Cu^{++} $3{\cdot}3\times10^{-4}$ M	Cu^{++} 10^{-4} M	Cu^{++} 10^{-5} M
120	11	70	116	90	11	48	86

Mitochondrial respiration. Experiments were carried out with mitochondria isolated from the livers of heliotrine-poisoned rats, with the following aims:

(i) To confirm the results of the homogenate experiments and localize the respiratory enzyme failure to the mitochondria.

(ii) To determine whether the pattern of failure of the individual systems agreed with that of the homogenate experiments.

(iii) To determine whether mitochondria isolated from liver in the 14- to 20-hour stage showed partial or complete restoration of activity by DPN addition to the medium.

(iv) To determine whether any latent defect was demonstrable in mitochondria isolated from animals killed prior to the phase of general respiratory failure.

The data of Table 5 show that the failure of homogenate respiration was duplicated in the mitochondria at similar times, and that a high degree of reactivation was obtained by addition of DPN to the medium.

TABLE 5.

Effect of DPN on the respiration of freshly isolated liver mitochondria from control and heliotrine-poisoned rats.

System as described in Methods. Oxygen uptakes (μl.) by liver mitochondria/gm. body wt./½ hr., without DPN addition (first figure) and with DPN addition to the medium.

Animal	Citrate		α-keto-glutarate		Malate		Glutamate		β-hydroxy-butyrate		Choline	
Control (3 expts.)	49	57	40	45	33	33	41	50	28	28	41	52
10 hr. heliotrine (2 expts.)	45	53	37	42	32	33	36	44	26	26	38	45
17 hr. heliotrine (2 expts.)	4	40	5	37	2	15	4	48	1	17	32	42

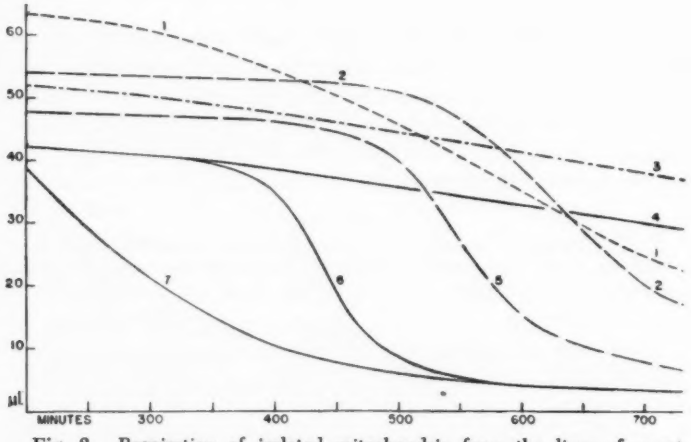

Fig. 3. Respiration of isolated mitochondria from the liver of a rat killed 14 hours after heliotrine administration when "aged" at 0° C. Explanation in text. Data from Table 6. Continuous lines: oxygen uptakes in the absence of added DPN, with citrate (7) α-ketoglutarate, glutamate, malate (6) and choline (4). Interrupted lines: oxygen uptakes in the presence of added DPN, with citrate (1) α-ketoglutarate (5), glutamate (2) and choline (3).

TABLE 6.

Changes in enzyme activity of rat liver mitochondria from 14 hr. Heliotrine rats on "Ageing" at 0°C. in the absence of substrate, and effect of DPN.

System as described in methods. Oxygen uptakes (μl.) by liver mitochondria/gm. body wt./½hr., without DPN addition to the medium (first figure) and with DPN.

Minutes of ageing (from death of animal)	Citrate		α-keto-glutarate		Malate		Glutamate		β-hydroxy-butyrate		Choline	
120	49	65	41	48	34	35	42	53	26	26	42	54
280	24	61	41	45	33	34	40	52	23	24	41	53
400	10	54	43	47	31	37	41	50	21	23	39	53
520	6	44	—	—	—	—	—	—	—	—	37	46
560	4	40	5	37	2	15	4	49	1	17	33	43
740	2	22	2	6	3	12	1	17	1	12	29	37

Table 6 (shown graphically as Fig. 3) indicates that a latent mitochondrial defect was present shortly before the phase of decline of respiratory activity began. In this experiment, mitochondria from a 14-hour heliotrine-poisoned animal showed activity at control levels when freshly isolated.

After "ageing" at 0° C. in the absence of substrate, normal mitochondria show no DPN reversible fall of activity for at least 10 hours, but with the 14-hour preparation the oxidation of malate, glutamate and α-ketoglutarate (curve 6) began to fall off rapidly 400 minutes after death of the animal, and by 500 minutes, the utilization of these substrates was negligible, although control preparations were still showing undiminished activity. From curves 2 (glutamate) and 5 (α-ketoglutarate) it will be seen that this loss of activity on ageing was, at this stage, still reversible by the addition of DPN. Results with malate were similar, the curve resembling that of α-ketoglutarate (curve 5). Choline oxidation (curve 4) did not fall greatly during the ageing period and the effect of DPN addition (curve 3) was small. Citrate oxidation (curve 7) readily became DPN deficient but responded to DPN addition for a prolonged period (curve 1).

DISCUSSION.

An attempt has been made to investigate some of the biochemical changes occurring in liver cells after administration of heliotrine, a member of a group of selectively hepatotoxic alkaloids.

The use of pathological material involves certain special problems, especially those of obtaining uniformity of action of the agent on all the susceptible cells, and from animal to animal. An appropriate reference standard for comparison of enzyme activities between animals is also necessary.

Thus a single high dose, 320 mg./kg. body wt. by intraperitoneal injection was used to obtain, as nearly as possible, a maximum effect on every liver cell simultaneously. This can never be achieved in practice with an agent which produces a zonal effect on the hepatic lobule. In these circumstances bio-

chemical methods demonstrate an "average state" inclusive of cells affected to varying degrees.

However, if the properties of the agent permit the use of a single dose sufficiently high to destroy almost every liver cell in every animal tested, the biochemically estimated "average stage" at any time may reasonably be regarded as reflecting one of the stages through which the individual cells must pass. The rapid course, resulting in extensive necrosis within 24 hours, also eliminates the possibility of side effects due to regenerative processes, which in any case are negligible with heliotrine.

In spite of strict precautions to ensure uniformity of strain, sex, age and diet of the animals, the evolution of the lesion was slower in some animals by as much as 4-6 hours in 20 hours. However, the pattern of the histological and biochemical findings was unchanged.

A reference standard is required when comparing enzyme activities or other analytical data, of a particular sample of liver tissue, with those of other samples.

The standards commonly used with normal tissue (wet-weight, dry-weight, and total nitrogen) were obviously valueless with pathological material of this kind (1. Histological Changes). DNAP was satisfactory for carbon tetrachloride poisoning (Christie and Judah, 1954), but the present results showed that it was not applicable with heliotrine poisoning.

The method tried, and eventually adopted as satisfactory, was to refer the measured gas exchange over a standard period to the quantity of liver tissue present in the animal per gram of body weight. This implies that in spite of the variations of liver weight and composition under normal and pathological conditions, the total liver activity of a particular enzymatic function is more consistently proportional to the body weight than to the liver weight, nitrogen content, or even to the number of cells present.

It was shown (Fig. 1) that a fall in the ratio

$$\frac{\text{liver wt./100 gm. body wt. (heliotrine-poisoned)}}{\text{liver wt./100 gm. body wt. (control)}}$$

occurred between the 2nd and 11th hours. This indicates that an initial loss of liver weight took place in the heliotrine-poisoned animals. The histological observations showed that necrosis was not extensive enough at this time to account for the weight loss, which must therefore have been mainly due to loss of the cytoplasmic basophilic material. Loss of liver weight by this process must have been opposed by progressive fluid imbibition and small vacuole fatty change, so that by the 11th hour the fall was arrested. Liver weight then began to increase, and continued to do so until the animal died.

After 15 hours, progressive haemorrhage into the necrotic tissue must have become a major factor (1. Histological Changes) opposing the decrease of bulk of hepatic tissue by shrinkage and by washing away of cell fragments into the sinusoids.

In the homogenate experiments, no explanation could be given for the early fall of aerobic pyruvate and octanoate utilization.

The enzymes of the intermediate group were all DPN dependent, and of high activity. Three components of the tricarboxylic (TCA) cycle were included in the group examined.

The sudden and complete loss of activity of these enzymes in the homogenate experiments coincided with a state of generalized, severe, cytoplasmic and nuclear changes in, on microscopic examination of, the livers (1. Histological Changes). When placed in the histological sequence, they corresponded to the stage immediately preceding general necrosis.

It may thus be concluded that when the DPN dependent enzymes lost activity, and the TCA cycle thereby became disorganized, cell necrosis rapidly followed. The failure of aerobic energy metabolism was therefore a late effect of heliotrine poisoning, preceded for many hours by obvious histological changes. Although of great importance as the immediate factor precipitating necrosis, it was unlikely to be the primary action of the drug on the liver cell.

With succinoxidase, and also with choline oxidase although DPN dependent, there was no sudden complete loss of activity at the 16th hour, but only the beginning of a gradual fall. Even as late as the 28th hour, their activities were still almost two-thirds of the normal level.

Histological examination and DNA analysis (Table 1) showed that at least one-third of the liver cells had completely disappeared at this time, and that most of the remaining tissue was necrotic. It must, therefore, be concluded that much of the oxidation of both these substrates at this time was being carried out by necrotic tissue.

The mitochondrial experiments showed that even after the decline of TCA cycle enzymes had disrupted organized TCA cycle activity, partial or complete reactivation of the component enzymes could be obtained if DPN were present in the medium.

This had previously been observed with rat liver after carbon tetrachloride poisoning (Christie and Judah, 1954), and it was postulated that mitochondrial damage caused the release of DPN from the enzymatic foci. Activity of this group of enzymes was also lost by liver from normal rats if allowed to "autolyse" at room temperature after death (Gallagher, Judah and Rees, 1956) and could be restored by DPN addition to the medium.

Schneider (1948) showed that isolation of mitochondria by centrifugation must be carried out at temperatures of the order of 0°-3° C. if satisfactory oxygen uptakes were to be obtained.

Ernster (1956) investigated the loss of activity of mitochondria from normal rat liver when exposed to room temperature in the absence of substrate. Activity could be restored by addition of DPN to the medium, and the reactivated preparation was still capable of esterifying inorganic phosphate.

Siekevitz and Potter (1955) directly demonstrated that mitochondria rapidly lost adenine nucleotides, including DPN, when exposed to 30° C. without substrate, but even after 10 hours at 0° C. negligible losses occurred. This is in agreement with the finding that DPN addition does not greatly increase the oxygen uptake of liver mitochondria from normal rats even after "ageing" at 0° C. for 10 hours.

The present results indicate that a major biochemical effect of heliotrine on the liver cell – although a relatively late one from the histological standpoint – is to determine a DPN "deficiency" of certain mitochondrial DPN dependent enzymes, which leads to TCA cycle disorganization, and the rapidly ensuing necrosis. This DPN "deficiency" effect could be due to actual DPN leakage as in "ageing", or to DPN inactivation *in situ*, or to defective DPN synthesis or supply to the enzyme centres.

The attempts to demonstrate a latent mitochondrial defect by "ageing" at 0° C. were not successful until a time (14th hour) shortly before overt loss of respiratory activity would have been expected.

The respiratory changes were not specific to heliotrine toxicity, but were very similar to those found in poisoning by carbon tetrachloride and dimethylnitrosamine (Bailie and Christie, unpublished) and also in anoxia (Gallagher, Judah and Rees, 1956). That TCA cycle disorganization due to coenzyme deficiency has been observed with such diverse agents indicates that it may be a common terminal phenomenon in acute severe toxicity in which there is no specific inhibition of respiratory metabolism.

It must be admitted that very little evidence can be offered concerning the earlier stages of heliotrine poisoning. Prior to the failure of energy metabolism, retrogressive changes were present histologically in a high proportion of the nuclei, and the nuclear fraction when isolated by differential centrifugation was abnormally swollen, red and gelatinous. Bull (1955) has drawn attention to the greatly enlarged nuclei in chronic poisoning by pyrrolizidine alkaloids. Cytoplasmic vacuolation by material stainable by Weigert's fibrin method and loss of cytoplasmic basophilia also precede the respiratory changes. On the basis of all the findings, it is suggested as a working hypothesis that the alkaloid may have a primary effect on some cell constituent such as a protein, possibly even a nucleoprotein, and that this is eventually reflected in the respiratory changes.

SUMMARY.

Some biochemical changes occurring in rat liver in acute poisoning by the hepatotoxic alkaloid heliotrine have been described.

After an initial fall, maximum at the 10th hour, the liver weight per 100 gm. body weight progressively rose.

Loss of activity of DPN dependent respiratory enzymes and disorganization of the TCA cycle occurred suddenly at the 16th hour. This was initiated by "lack" of mitochondrial DPN. Extensive histological necrosis rapidly followed.

Succinoxidase and choline oxidase activity and anaerobic glycolysis persisted at an unexpectedly high level after histological necrosis was well advanced.

In comparing the activity of an enzyme in different animals, the reference standard adopted was the activity of that quantity (wet weight) of liver tissue present per gram of body weight per unit time.

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REFERENCES.

Bull, L. B. (1955): Austral. vet. J., 31, p. 33.
Christie, G. S., and Judah, J. D. (1954): Proc. roy. Soc. B., 142, p. 241.
Ernster, L. (1956): Exp. Cell Res., 10, p. 721.
Gallagher, C. H., Judah, J. D., and Rees, K. R. (1956): J. Path. Bact., 72, p. 247.
Keilin, D., and Hartree, E. F. (1937): Proc. roy. Soc. B., 122, p. 298.
Le Page, G. A. (1948): J. biol. Chem., 176, p. 1009.
Schmidt, G., and Thannhauser, S. J. (1945): *Ibid.*, 161, p. 83.
Schneider, W. C. (1948): *Ibid.*, 176, p. 259.
Schneider, W. C., and Potter, V. R. (1943): *Ibid.*, 149, p. 217.
Siekevitz, P., and Potter, V. R. (1955): *Ibid.*, 215, p. 221.

A STUDY OF THE PERIOD OF ACTION IN A SERIES OF SEX-LINKED RECESSIVE LETHALS IN *DROSOPHILA*

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Pre-imaginal development in *Drosophila* is controlled by a closely-integrated biochemical net-work, sensitive to any genetic or environmental modification in reaction velocity (Goldschmidt, 1938). When this interlocking system is subjected to genetically engendered physiological or morphological variations sufficiently aberrant to cause death before the attainment of sexual maturity, the inherited factor is known as a lethal. Qualitative studies on the embryonic expression of certain recessive lethals were published recently (Ede, 1956a, b, c, d) while deficiencies and Minutes played an important part in a detailed investigation into the chromosomal control of ontogenetic organisation (Poulson, 1937, 1940; Brehme, 1939; Kallis, 1939; Farnsworth, 1957a, b). The past five years have seen a revival of interest in the quantitative aspects of developmental mortality. The earlier works of Sivertzev-Dobzhansky (1927); Li (1927); Brehme (1937) and Brody (1940) were too restricted to provide decisive information on the presence or absence of a developmental sensitivity pattern, e.g., Brody's erroneous conclusions on "the infrequency of pupal lethals" and the "non-specificity" of lethal action. Lethal clusters at specific points in the ontogenetic cycle were first recognised by Medvedev (1939a, b) and subsequently confirmed by Hadorn (1948) who saw in this sensitive grouping the visible expression of periodic fluctuations in metabolic activity during development. The term "phase-specificity" adopted by Hadorn (1951) describes the regularity (within reasonably narrow limits) with which contemporaneous individuals bearing the same lethal factor cease development. Apparent deviations from this rule are regarded as the consequence of modification by the residual genotype or of pleiotropic debility causing a certain proportion of premature deaths by a reduction in the capacity to cope effectively with environmental selection.

Comparative surveys of lethals from various sources, viz., spontaneous or radiation induced, were made by Oster (1952, 1954), Rizki (1952) and Seto (1954), while a brief account of ultra-sonic induced lethals has also appeared (Shiomi, 1955). The present study is an attempt to detect any variation in the relative size of lethal groupings when the same X-ray dose is administered at two different intensities.

Materials and Methods.

Batches of wild type (Canton-S) *Drosophila melanogaster* males were treated with an X-ray dose of 2150r, delivered at one of the two alternative intensities, 2000r/min. (High) or 100r/min. (Low). Induced sex-linked recessive lethals were detected and maintained in permanent stock by the Muller-5 (M5) technique (Spencer and Stern, 1948). As part of a detailed programme on mutation, all lethals were also analysed cytologically and localised genetically by out-crossing the heterozygous M5/lethal females to Xple (y apr ec cv ct v f) males.

For experimental purposes, the developmental period in *Drosophila* may be divided into four somewhat artificial sections, determined in part by the technique adopted: (a) egg or embryonic—E, (b) early larval—EL—including the first instar and the early part of the second instar, (c) late larval—LL—the latter half of the second instar and the third instar, (d) prepupal and pupal stages combined—P. In each run, three M5/lethal females were pre-mated to three M5 males for one day. These flies were then placed in an empty half-pint milk-bottle inverted over and taped to a small Petrie-dish containing normal medium darkened with a trace of animal charcoal to provide optical contrast. Normally, the determinations were made in lots of six lethals, each lot having one control bottle. Egg-laying periods were restricted to a maximum of four, aiming at a viable progeny minimum of one hundred. To reduce residual mortality, the test flies were mated as soon as possible after emergence and to ensure a reasonable homogeneity in the ages of the off-spring, the duration of each egg-laying period seldom exceeded twelve hours. The first day's egg yield was invariably discarded to avoid a spurious "embryonic mortality" arising from the initial rapid deposition of unfertilised eggs. At the end of each twelve-hour laying period, when the eggs had been counted, the Petrie-dishes were transferred to a fly-proof incubator-box and left at 25° C. for thirty-six hours. At this point, the number of unhatched eggs was recorded. Twenty-four hours later, this figure was checked and all viable larvae placed in yeasted culture vials, a maximum of thirty per tube, to complete development. This collecting period marks the end of the early larval stage. Late larval lethality was detected by the relative proportion of collected larvae which failed to form puparia. Where possible, dead or moribund larvae were diagnosed as carrying the lethal X-chromosome by gonadial morphology and Malpighian tubule colour, while appropriate individuals were examined for visible abnormalities. In most cases, dead larvae could be aged accurately by the anterior spiracles and/or the dentition of the jaw hooks. Post-larval lethals were directly distinguishable in dissected aberrant pupae.

RESULTS AND DISCUSSION.

The results of this and related investigations are summarised in Table 1. A more detailed treatment of the individual lethal counts is available elsewhere (Strangio, 1957). Oster's (1952, 1954) material was selected for point mutations alone; for this reason, only Rizki's (1952) and Seto's (1954) data are sufficiently comprehensive for graphical representation and comparison with the present study (Fig. 1). Following the practice adopted by these authors, both early and late larval lethals are grouped into a single category. Occasionally, lethality may not be limited to a particular ontogenetic phase, but in the majority of such cases, a statistically rigid temporal restriction about the transition zone between two consecutive stages still applies, giving rise to the so-called boundary lethals (egg-larval E/L, larval-pupal L/P). The rare extended phase lethals, however, express their lethality over a wide range with no sharply defined limits.

TABLE 1.

Author	Organism	Type	Source	E	E/L	L	L/P	P	Total
Rizki, (1952)	*D. willistoni*	Autosomal	(a) Spontaneous	1	4	19	4	1	29
			(b) X-ray	6	3	7	0	3	19
Seto, (1954)	*D. melanogaster*	Autosomal	(c) X-ray	7	5	12	1	2	27
Strangio, (1957)	*D. melanogaster*	Sex-linked	(d) X-ray	12	5	37	7	7	68

Significance tests: *Significant at 5% level; ≠Not significant at 5% level.
(b) vs. (c) ≠; (b) vs. (d) ≠; (c) vs. (d) ≠; (a) vs. (b)*; (a) vs. (c) ≠; (a) vs. (d). ≠

Sex-linked, recessive lethals have been arranged into three groups depending on the appearance of the salivary gland chromosomes in the heterozygous wild-type/lethal female (Lea, 1955). The heterogeneous class, known as the "point" mutations, includes those lethals in which chromosomal damage, if present at all, does not exceed the resolution threshold of the light microscope, as well as undetectable single-band inversions (Group I). One- and two-hit deficiencies ranging in size up to approximately fifty chromatic bands act as Group II lethals (larger deletions presumably contribute to the dominant lethal fraction). Gross rearrangements — translocations and inversions — constitute Group III. A cytological study of nineteen spontaneous (Slizynski, 1938) and twenty-one ultra-violet induced (Slizynski, 1942) lethals revealed the predominance of point mutations and small deletions not exceeding fourteen bands, the latter being interpreted (Slizynska, quoted Slizynski, 1942) as single-event phenomena brought about by the impairment of intragenic function and the loss of self-reproducibility during a subsequent division cycle. The frequencies of these small deficiencies in X-ray, ultra-violet induced and spontaneous lethals are approximately equal but high-energy mutagenesis also promotes the formation of still larger deletions and gross chromosomal rearrangements through an increase in the probability of producing two contemporaneous, spatially adjacent breaks in the nuclear material. Emerging from a number of relevant investigations (Li, 1927; Demerec and Hoover, 1936; Poulson, 1945), a general proportionality can be seen to exist between the time of onset and the severity of ontogenetic abnormality on the one hand and the extent of lethal-associated

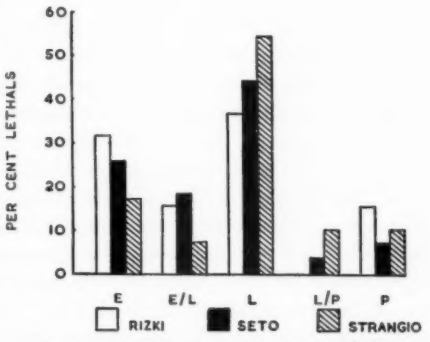

Fig. 1. Graphical representation of the relative mortality in each sensitive period from three independent studies of the time of action of X-ray induced lethals.

aberrations on the other (Groups II and III). An adequate assessment of the trend in spontaneous lethal mortality is available only from Rizki's work on autosomal recessives in *D. willistoni* (1952). If this is accepted as a valid basis for comparison with *D. melanogaster,* then all three independent studies of X-ray induced lethality show the expected (though not fully significant) drift towards embryonic and immediately post-embryonic stages as determined by the introduction of the developmentally more susceptible intergenic mutations (Table 1).

The original target concept of radiation mutagenesis as a direct and exclusively nuclear process (Lea, 1955) has met with increasing difficulties over the past decade. The first important challenge to the theory came with the discovery of a distinct "oxygen-effect" accompanying the irradiation of *Vicia faba* root-tips in air or pure nitrogen (Thoday and Read, 1947). Subsequently, it was discovered that in *Drosophila* the injection of azide or cyanide prior to irradiation leads to a significant increase in the number of recessive lethals produced by a given dose of irradiation (Sobels, 1955; Clark, 1956), presumably as a result of the inhibition of the catalase system. It is now reasonably certain that high-energy radiations directed on germ-cell tissue have, besides a direct physical action on the protein molecules of the chromosome string, a subsidiary, indirect effect in producing extra-nuclear chemical mutagens. The ultimate effectiveness of the active radio-chemical primaries depends on the concentration of chemical mutagen (whether it be peroxide radicals themselves or daughter compounds) which actually comes into contact with the chromosomes and this is probably determined to a large extent by the catalytic efficiency of the cell's catalase system.

Haas *et al.* (1954) observed a differential translocation yield following the irradiation of *D. virilis* at two different intensities and interpreted their results as follows. At the higher intensity, an almost instantaneous flood of the accessory chemical mutagens in the cytoplasm reaches a level far in excess of the upper limit of capacity in the protective mechanism, enabling a substantial portion to diffuse across the nuclear membrane before the catalytic protein becomes reavailable. The same absolute quantity of primary chemo-mutagen is released at the lower intensity, but the slower delivery rate ensures that at no time is its concentration as far above the tolerance threshold as in the preceding case, with the result that a much smaller volume reaches the sensitive area in the nucleus.

In the present study, it had been expected that an increased yield of gross aberrations at the higher dose rate would be mirrored in the more obvious embryonic trend in the sensitivity pattern of these lethals; but no significant difference between the high and low dose-rate series was apparent (Table 2). However, this is not to be taken as convincing proof that a change of some kind has not occurred. With the assumption that at 2000r, approximately 16 p.c. of

TABLE 2.

Intensity	E	E/L	L	L/P	P	Total
(a) High	6	3	13	4	5	31
(b) Low	6	2	24	3	2	37

Significance test: (a) vs. (b) $\neq$

the total low intensity lethals scored are associated with gross chromosomal abnormalities (Catcheside, 1948), then, even after allowing for half as many structural rearrangements again induced at the higher intensity (Haas' figures actually suggest an increase of only one-third at 2000r), a marked difference might still remain undetected in a survey of this size.

As in Rizki's data, a number of lethals were detected which exhibit in balanced stocks higher mortality rates than the expected 25 p.c., while several extended phase lethals were also found. Excess lethality may be due to (a) the presence of two (or more) lethals on the one chromosome far enough apart for frequent crossing over to cause a considerable separation of the individual components in the lethal "tandem", (b) semi-dominance of the lethal, and (c) association of the lethal with a translocation. In the present work, the use of the M5-crossover suppressor insures against error introduced by a multi-lethal chromosome. Partial dominance may be checked by a statistical analysis of any deviation from the 2:1 female-male ratio. Direct cytological observation provides a means of distinguishing between the true lethal effect usually induced at one breakage point and the residual embryonic mortality following the abnormal segregation of translocated chromosomes. The simplest explanation for extended phase lethals interprets their lack of specificity as the result of genic or environmental modification (Hadorn, 1951). Alternatively, a multi-locus effect is suggested on the basis of the mortality pattern of *lethal* (80), a large deficiency of approximately 48 bands (Fig. 2 and Table 3). By inference from the nature of the developmental anomalies induced by deficiencies of varying size, Poulson (1945) was able to pinpoint chromosomal segments responsible for normal tissue differentiation during embryogenesis. Obviously, the chance of excluding a character necessary for normal ontogeny would be improved by increasing the size of the deletion so that he was able to state with

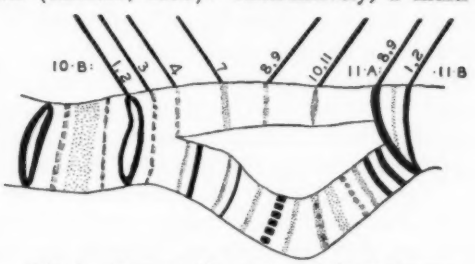

Fig. 2. Diagram of a portion of the X-chromosome from the salivary glands of an individual heterozygous for the *lethal-80* deficiency: breakage points in the vicinity of 10·B:10,11 and 11·A:8,9.

TABLE 3.

No.	Comments.	No.	Comments
36	Larval integument tanned but no recognisable puparium formed.	76	Similar indications of ring-gland failure.
42	Similar failure to pupate.	79	Abnormal pupation.
44	1st instar larvae survive up to 96 hours after eclosion.	80	Extended phase. Large deficiency. Individuals survive till late pupal stage; bristles irregular, abdomen telescoped, scutellum compressed in antero-posterior plane—now somewhat oblong in shape.
55	Early pupal lethal. Some reach stage of complete external differentiation—approximately 40 hours after puparium formation. Appendages deformed.	83	Extended 3rd instar.
58	Early prepupal. Dark-brown pigment ring in region of operculum.	88	Small 3rd instar larvae. Extended larval stage, finally climb walls of tube, contract but do not become tanned. Rudimentary brain and imaginal buds.
67	Extended phase. 3rd instar larvae superficially normal but persist 8-9 days after normal culture-mates have pupated. Majority die as larvae, some form aberrant pseudopupae. Dissected larvae have sparse fat-body, rudimentary imaginal buds, cerebral ganglia and ring-glands.	97	3rd instar extended.
		99	Prepupal lethal. Puparia elongate and abnormal.
		109	Delay in pupation.

some degree of certainty that, for large deficiencies, "the effects are early and extensive". In *lethal* (80), however, there is no indication of phasic restriction, death occurring in all stages, and even some fully developed flies making feeble, though ineffectual, attempts to escape from the pupal case. It would appear that in this instance, the missing chromosomal fragment conforms to Muller's (1932) concept of hypomorphic character – loss of function without being sufficiently drastic to cause death, but having in association a general lowering in vitality, i.e. *l*(80), is probably no more than a synergistic sub-lethal cluster. A certain fraction succumbs to a detrimental unit at one stage while the survivors which would normally reach maturity, now face further decimation as another sub-lethal field comes into operation at a later sensitive period and so on, until ultimate summation eliminates the class as a whole.

Listed in Table 3 are the results of the microscopical examination of the more obviously aberrant types. Larvae which appeared generally reduced in size but with no additional pronounced morphological change are omitted. Bodenstein's (1944) work on the hormonal control of moulting in *Drosophila* emphasised the importance of an even growth-differentiation balance during development. The basic ramifications of the hormone net-work and its points of contact with other post-embryonic and pre-imaginal systems remain a closed book, but just how far-reaching and sensitive are the radiating links, is reflected in the comparatively large number of lethals which have lost the ability to moult

or pupate. A problem arises in assigning this loss of function to its exact point of origin – abnormality in the neurosecretory cells of the corpus allatum and/or associated organs, production of an aberrant hormone (cf. sickle cell haemoglobin) or the absence of a specific activator. In many cases, direct cytological study reveals conditions bearing some resemblance to those found in "lethal-giant" by Scharrer and Hadorn (1938). The survival of these larvae 7-20 days after their normal sibs have pupated and the occasional formation of pseudo-pupae led these authors to conclude that the rudimentary ring-glands were secreting normal hormone but at a much slower rate. The threshold level necessary for the onset of pupation was rarely, if ever, reached. In other cases, however, the glands are superficially normal. Experiments to gauge the potentialities of these "lethal" ring-gland complexes are being carried out with reciprocal transplants between wild-type and abnormal larvae.

SUMMARY.

A study of approximately seventy induced lethals was made to determine the ontogenetic distribution of mortality. The results obtained in the X-ray group tally with other published estimates. The usual clustering phenomena were observed as well as a small number of extended phase lethals. An attempt was made to interpret the latter as the expression of multiple sub-units.

No significant difference was noted between the sensitivity patterns of lethals induced at high or low X-ray intensities.

Moulting abnormalities were common. The point at which the biochemical block in hormone production operates is uncertain, but it seems most probable that more than one site is involved. Further work is in progress.

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REFERENCES.

Bodenstein, D. (1944): Biol. Bull., 86, p. 113.
Brehme, K. S. (1937): Amer. Nat., 71, p. 567.
Brehme, K. S. (1939): Genetics, 24, p. 131.
Brody, G. (1940): *Ibid.*, 25, p. 643.
Catcheside, D. G. (1948): Adv. in Gen., 2, p. 271.
Clark, A. M. (1956): Nature, 177, p. 787.
Demerec, M., and Hoover, M. E. (1936): J. Hered., 27, p. 207.
Ede, D. A. (1956a): Arch. Entw.-Mech. Org., 148, p. 416.
Ede, D. A. (1956b): *Ibid.*, 148, p. 437.
Ede, D. A. (1956c): *Ibid.*, 149, p. 88.
Ede, D. A. (1956d): *Ibid.*, 149, p. 101.
Farnsworth, M. W. (1957a): Genetics, 42, p. 7.
Farnsworth, M. W. (1957b): *Ibid.*, 42, p. 19.

Goldschmidt, R. B. (1938): Physiological Genetics, New York, McGraw-Hill.
Haas, F., Dudgeon, E., Clayton, F., and Stone, W. (1954): Genetics, 39, p. 453.
Hadorn, E. (1948): Symp. Soc. exp. Biol., 2, p. 177.
Hadorn, E. (1951): Adv. in Gen., 4, p. 53.
Kallis, N. (1939): Genetics, 24, p. 244.
Lea, D. E. (1955): Actions of Radiations on living Cells, 2nd ed., Camb. Univ. Pr.
Li, J. C. (1927): Genetics, 12 p. 1.
Medvedev, N. N. (1939a): C.R. acad. sci. U.S.S.R., 22, p. 344.
Medvedev, N. N. (1939b): *Ibid.*, 25, p. 517.
Muller, H. J. (1932): Proc. 6th Int. Cong. Genet., 1, pp. 213-255.
Oster, I. I. (1952): Heredity, 6, p. 403.
Oster, I. I. (1954): Nature, 173, p. 594.
Poulson, D. F. (1937): Proc. Nat. Acad. Sci. (U.S.), 23, p. 133.
Poulson, D. F. (1940): J. exp. Zool., 83, p. 271.
Poulson, D. F. (1945): Amer. Nat., 79, p. 340.
Rizki, M. T. M. (1952): J. exp. Zool., 121, p. 327.
Scharrer, B., and Hadorn, E. (1938): Proc. Nat. Acad. Sci. (U.S.), 24, p. 236.
Seto, F. (1954): J. exp. Zool., 126, p. 17.
Shiomi, T. (1955): Drosophila Information Service, 29, p. 164.
Sivertzev-Dobzhansky, N. P. (1927): Arch. Entw.-Mech. Org., 109, p. 535.
Slizynski, B. M. (1938): Genetics, 23, p. 283.
Slizynski, B. M. (1942): Proc. Roy. Soc. (Edin.), B61, p. 297.
Sobels, F. H. (1955): Z. ind. Abstr. Ver., 86, p. 399.
Spencer, W. P., and Stern, C. (1948): Genetics, 33, p. 43.
Strangio, V. A. (1957): Unpublished Thesis, Univ. of Melbourne.
Thoday, J. M., and Read, J. (1947): Nature, 160, p. 608.

HEXOKINASE ACTIVITY OF SHEEP, LAMB AND RAT TISSUE

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In some respects the carbohydrate metabolism of young lambs resembles that of non-ruminating animals, and so differs from that of the adult sheep (McCandless and Dye, 1950; Jarrett and Potter, 1952).

In the young lamb, the response to an injection of insulin, and both the duration of hyperglycaemia and the rise in the level of blood pyruvate that follow an intravenous injection of glucose, are different from those observed in adult sheep (Jarrett and Potter, 1952). When glucose is administered into the abomasum of adult sheep there is a slow rise in the level of sugar in the blood; with a young lamb, however, a rapid rise in blood sugar occurs after ingestion of a glucose solution (Reid, 1950; Jarrett and Potter, 1952).

It seems possible then that the lower capacity of the adult sheep to utilize glucose may be due to an impairment of the hexokinase system.

Many observations have been made to indicate an effect of previous diet on various aspects of carbohydrate metabolism (Himsworth, 1935; Samuels, Gilmore and Reinecke, 1948; Garner and Roberts, 1955). Long (1953) has shown that by feeding diets of high or low fat content changes can be produced in the hexokinase activity of the small intestine of rats.

In view of the novel characteristics of the alimentary tract of ruminants and the production and absorption of large amounts of lower fatty acids in these animals, a study was made of the hexokinase activity of sheep and lamb tissue and this was compared with the activity of rat tissue.

The principle of the method depends upon the phosphorylation of glucose to glucose-6-phosphate by a buffered homogenate of tissue in the presence of adenosine triphosphate and certain ions. The rate of disappearance of free glucose from the system was interpreted as an indication of the hexokinase activity of the tissue.

EXPERIMENTAL.

The adult sheep used in these experiments were merino ewes and wethers aged 3-5 years and fed on either 800 gm. of wheaten hay chaff or on fresh green pasture. The lambs were from ewes grazed on pastures. Six unsuckled lambs less than 24 hours old, ten lambs between 12 hours and 5 days old and eleven lambs between 6 and 35 days were used.

Eighteen male rats in three groups of six were placed at the time of weaning (one month old) on a basal diet, a high-fat diet and a fat-free diet (Long, 1953). Observations on their tissues served as a basis of comparison for the observations with sheep and lambs.

As the laboratory colony rats fed on a standard stock ration showed a better growth rate than those on the diets of Long (1953) a number of these animals were used also for determination of hexokinase activity of intestinal mucosa and brain.

The animals were killed by decapitation and a length of duodenum and small intestine was removed and placed in ice-cold water. The intestine was cut longitudinally, carefully washed and blotted, and the mucosa scraped off. Approximately 0·5 gm. was homogenized for one minute in 4 ml. of ice-cold buffer using a Potter-Elvehjem glass homogenizer. One gm. of mucosa was used for determination of defatted dry weight. The tissue was dried in an oven at 110° C. for 24 hours and then defatted by continuous extraction with ether for one hour. Samples of brain were treated similarly.

Two types of buffer were used, an 0·12 M. potassium phosphate buffer, pH 7·8, containing 0·15 M. potassium fluoride (Long, 1951) and a veronal buffer pH 8·0 containing 0·07 M. veronal and 0·029 M. potassium fluoride. With the tissue from rats on the diets of varying composition the technique of Long (1953) was followed closely, but with sheep and lambs the veronal buffer was found to give slightly better results and was used for all subsequent work. The final composition of the homogenate was 0·5 gm. of tissue, 0·062 M. sodium veronal, 0·026 M. potassium fluoride in a total volume of 4·5 ml.

The disodium salt of adenosine triphosphate (Pabst) was made up to pH 7·6 as an 0·052 M. solution and stored in deep freeze. The glucose solution was made up fresh each day by adding glucose to a magnesium chloride-potassium chloride mixture giving a solution containing 0·025 M. $MgCl_2$, 0·210 M. KCl and 0·017 M. glucose.

The activity of the tissue at three concentrations of homogenate was determined by placing in each of four tubes in a water bath at 30° C. 0·1 ml. ATP solution, 0·1 ml. KCl-$MgCl_2$-glucose solution and to each of the tubes respectively 0·3 ml. buffer; 0·2 ml. buffer + 0·1 ml. homogenate; 0·1 ml. buffer + 0·2 ml. homogenate; 0·3 ml. homogenate. The composition of the test system was therefore approximately 10, 20 or 30 mg. of tissue, 0·037 M. veronal, 0·016 M. KF, 0·005 M. $MgCl_2$, 0·042 M. KCl, 0·003 M. glucose, 0·010 M. ATP. Blanks were determined on each concentration of homogenate by using water instead of ATP. The system was incubated for ten minutes. Enzymatic reaction was stopped by adding 0·5 ml. 0·3N. $Ba(OH)_2$ and the protein and glucose-6-phosphate precipitated by addition of 0·5 ml. 5 p.c. $ZnSO_4$. After adding 3·5 ml. water and shaking, the solution was centrifuged and filtered through Whatman No. 42 papers and the free glucose determined by the method of Nelson (1944) and Somogyi (1952).

The hexokinase activity was expressed as mg. glucose utilized in 10 minutes by 1 mg. of defatted dry tissue.

RESULTS.

Although the amounts of glucose utilized by rat intestinal mucosa reported here differ slightly from those obtained by Long (1953) differences between animals on two of the diets have been confirmed. An analysis of variance was done on the results and a statistically significant difference was shown between the fat-free and high-fat diets and between the basal and high-fat diets. None of the dietary regimes effected any significant change in the hexokinase activity of brain tissue.

The rats had been kept on the diets for nine weeks after weaning and the growth curves showed the same trend as reported by Long (1953). The rate of growth, however, was slower than with animals on the colony diet, and to counteract any possible deficiency of vitamin B_{12} an intramuscular injection was given to half of the animals in each group after they had been on the diets for five weeks. The graphs in Fig. 1 represent the mean growth rate of two groups of three animals on each of the diets shown. The means for the animals on the basal diet were between those of the high-fat and fat-free group and are not plotted in Fig. 1. The divergence of the average values for the two groups of rats on the high-fat, fat-free and basal diets indicates that a weekly injection of 5 μg. of vitamin B_{12} did produce a growth response. No difference, however, could be detected between the two groups on the colony diet.

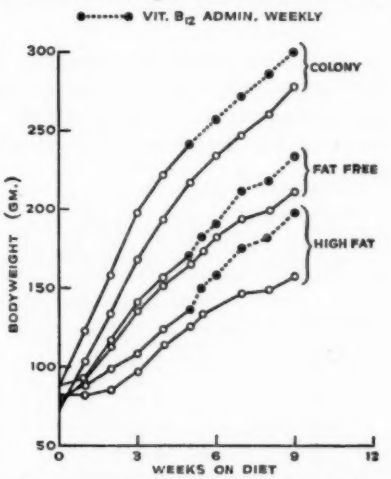

Fig. 1. Growth curves of rats showing effect of vit. B_{12} on the mean weights of each group.

Although a significant difference in the hexokinase activity of intestinal mucosa was apparent between rats on a high-fat and a fat-free diet and on a high-fat and basal diet there was no evidence to indicate that the administration of vitamin B_{12} had any effect on the activity of any group. The results are tabulated in Table 1. The activity was calculated as mg. of glucose utilized by 1 mg. of defatted dry tissue/hour from a determination on 0·2 ml. of homogenate. As a linear relationship was not found for amounts of rat tissue up to 0·3 ml. of homogenate these values based on a determination done at one concentration of 0·2 ml. of homogenate could not be used for a comparison between rat and sheep tissue. Consequently a group of colony rats were used and the activity of the tissue determined at

three concentrations of homogenate under the same conditions as with sheep and lambs. The amounts of glucose utilized at the three concentrations were plotted and the values for 1 mg. of defatted dry tissue/10 minutes obtained from the graph.

TABLE 1

Effect of diet on hexokinase activity of rat intestinal mucosa.

Mg. glucose utilized/Mg. defatted dry tissue/Hr.

Fat Free	Basal	High Fat
0·118	0·114	0·103
0·104	0·113	0·111
0·135	0·102	0·092
0·126	0·124	0·098
0·113	0·123	0·070
0·127	0·109	0·104
Mean	Mean	Mean
0·121	0·114	0·096

Least difference between means significant at the 5 p.c. level is 0·015.
The first three animals of each group received 5μg. Vit. B_{12} intramusc. once/wk.

Using this method of assessing hexokinase activity a mean value of 0·051 mg. glucose utilized/mg. defatted dry tissue/10 minutes was obtained for rat intestinal mucosa. A curve typical of the results obtained with rat tissue is shown in Fig. 2 and the values tabulated in Table 2.

TABLE 2.

Hexokinase activity of lamb, sheep and rat intestinal mucosa.

Mg. glucose utilized/Mg. defatted dry tissue/10 Min.

Unsuckled Lamb	½-5 Days Old Lamb	6-35 Days Old Lamb	Adult Sheep	Rat
0·020 (½)	0·027 (½)	0·015 (6)	0·017	0·045
0·017 (½)	0·025 (½)	0·022 (7½)	0·022	0·054
0·016 (½)	0·024 (2½)	0·016 (11)	0·020	0·060
0·014 (1)	0·021 (2)	0·026 (10)	0·020	0·048
0·014 (1)	0·024 (1)	0·016 (9)	0·020	0·049
0·021 (1½)	0·023 (3)	0·022 (14)	0·016	0·049
	0·022 (3)	0·022 (14)	0·015	
	0·025 (4)	0·018 (14)	0·016	
	0·028 (5)	0·018 (15)		
	0·024 (5)	0·013 (21)		
		0·023 (35)		
Mean	Mean	Mean	Mean	Mean
0·017	0·024	0·019	0·018	0·051

(Age in days shown in brackets)

Difference between lamb 1-5 days old and adult, $p. < 0·001$;
and between lamb 1-5 days old and 6-35 days old, $p. < 0·01$.

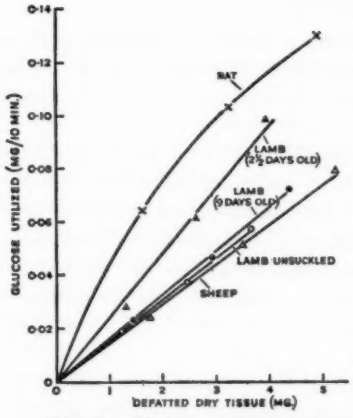

Fig. 2. Glucose utilization by intestinal mucosa.

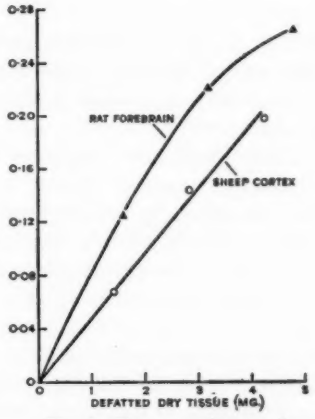

Fig. 3. Glucose utilization by brain tissue.

Eight adult sheep and 27 lambs were used for determination of the activity of intestinal mucosa. With both sheep and lambs a linear relationship was observed for the three concentrations of homogenate used (up to 5 mg. defatted dry tissue). A mean value of 0·018 mg. glucose utilized/mg. defatted dry tissue was obtained for sheep intestine. Results are tabulated in Table 2 and a graph typical of all animals is shown in Fig. 2.

With the lambs a difference in activity was noted depending on the age of the lamb. A mean value of 0·017 mg. glucose utilized was obtained with six unsuckled lambs less than 24 hours old. Two of these lambs had been given water by mouth and two a lactose solution, but no difference was detected due to this treatment. A mean value of 0·024 mg. glucose utilized was obtained with ten lambs between 12 hours and 5 days old and a value of 0·019 mg. glucose utilized with eleven lambs 6-35 days old. These results are presented in Table 2 and typical graphs shown in Fig. 2.

An analysis of variance indicated no statistical difference between new-born unsuckled lambs, lambs 6-35 days old and adult sheep. Lambs between the age of 1-5 days, however, did show a greater hexokinase activity of intestinal mucosa than lambs 6-35 days old ($p < 0{\cdot}01$) and adult sheep ($p < {\cdot}001$).

The hexokinase activity of cerebral cortex from the brains of five sheep and one lamb, and of brain stem (medulla) from two sheep, was determined. A mean value of 0·044 mg. glucose utilized was obtained with cortex of sheep and a value of 0·041 mg. with the lamb. The values for the medulla from the two sheep were 0·035 and 0·038 mg. respectively. Rat cerebral hemispheres from four rats gave a mean value of 0·080 mg. glucose utilized.

DISCUSSION.

Although it was not possible to obtain a linear relationship with rat tissue the graphs plotted in Figs. 2 and 3 show that significant differences occur between rat and both sheep and lamb tissue within the range of concentration of tissue used.

From the results it is apparent that the ability of both sheep brain and intestinal mucosa to utilize glucose is much less than that of the rat when compared on a defatted dry weight basis. With lack of information, however, on the specific activity in relation to the surface area exposed in the intact mucosa, it is a fallacy to apply these observations too closely to the overall activity in the whole animal. Nevertheless, these observations can provide further evidence for the assumption that in some aspects of the intermediary metabolism, sheep differ from non-ruminating animals.

The results also offer further support for the suggestion that the carbohydrate metabolism of the young lamb may differ to some extent from that of the adult sheep (McCandless and Dye, 1950; Jarrett and Potter, 1952). With the onset of suckling, the ingestion of colostrum, and a rise in blood glucose (Jarrett and Potter, 1952) there is apparently a concomitant rise in hexokinase activity of intestinal mucosa. While the elevated blood sugar persists for some weeks in a suckling lamb the hexokinase activity of intestinal mucosa declines after the first week of life and gradually assumes the level characteristic of adult sheep.

Apart from the overall activity, another important difference between rat and sheep tissue is related to the utilization of glucose depending on the concentration used. Long (1951) has claimed that there was a linear relationship with concentrations of homogenate lower and higher than that used in his standard test system. According to his data, however, there was some reduction in the rate of activity as the concentration of homogenate increased. For this reason all experiments reported here except those done as a repetition of Long's experiments (1953) were performed with three levels of concentration. It would appear that some inhibition was occurring with rat tissue at higher concentrations whereas with sheep and lamb tissue a linear response was obtained indicating optimum conditions for the system over the range used.

Various factors are known to inhibit the hexokinase reaction, amongst these being glucose-6-phosphate (Crane and Sols, 1954). In view of the low activity of sheep tissue it may be that the accumulation of end-product was not a limiting factor as it may have been with rat tissue. Although a number of factors (concentration of ions, ATP, glucose and buffer) were varied, it was not found possible to obtain a linear response with rat tissue.

Long (1953) showed that the activity of intestinal mucosa can differ depending on the diet, but no difference was found for intestinal muscle. The results reported here confirm his conclusion with mucosa and are extended to

show that no difference occurs in the activity of brain from rats on high-fat and fat-free diets. A similar difference was found between sheep brain and intestinal mucosa as between rat brain and mucosa, the brain activity being about twice that of mucosa. In view of the known pattern of variations in enzyme activity in different parts of the brain (Elliott, 1955), it is interesting to report that the activity of the medulla of the two sheep examined showed a slightly lower value than the cerebral cortex.

It is apparent that the nature of the diet can influence certain aspects of carbohydrate metabolism, and in view of the work of Long (1953) and of Garner and Roberts (1955) on lowered carbohydrate utilization in rat and hamster tissue when animals are fed a high-fat diet it is interesting to speculate on the low activity of tissues from sheep.

Considering the high carbohydrate intake of the very young lamb and its growing dependence on lower fatty acids as it becomes older, it is possible that this change has some bearing on the hexokinase activity herein reported.

However, although the change from a predominantly milk diet to one in which increasing amounts of cellulose and other polysaccharides provide the main bulk takes place early in life, no specific change of any biochemical reaction has been directly implicated in this trend. Reid (1953), for example, has shown that the change in blood sugar which occurs as the lamb becomes older is related to a disappearance of glucose from the red blood cell and he would not support the hypothesis of McCandless and Dye (1950) that this blood sugar change is associated with development of an active rumen fermentation.

Thus, while the observations reported here on isolated tissue support the contention that various aspects of the carbohydrate metabolism of the young lamb and of non-ruminants differ from those of adult sheep, the evidence is still inconclusive regarding the importance of differences in specific enzyme systems which may be peculiar to the digestion of ruminants.

SUMMARY.

The glucose utilization of rat, lamb and sheep tissue has been studied.

When compared on the basis of glucose utilization/mg. defatted dry tissue the intestinal mucosa and brain of rat has a greater activity than either sheep or lamb mucosa and brain respectively.

The activity of the intestinal mucosa from suckling lambs ½ day to 5 days old is higher than mucosa from either adults, new-born unsuckled lambs, or lambs 7 days to 35 days old.

Although a direct interpretation to the whole animal is not warranted, it is considered that the results can provide further evidence to show that differences do occur in the intermediary metabolism of carbohydrate between young lambs, adult sheep and non-ruminating animals.

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REFERENCES.

Crane, R. K., and Sols, A. (1954): J. biol. Chem., 210, p. 597.
Elliott, K. A. C. (1955): "Neurochemistry", Charles C. Thomas, Illinois, U.S.A.
Garner, R. J., and Roberts, R. (1955): Biochem. J., 59, p. 224.
Himsworth, H. P. (1935): Clin. Sci., 2, p. 67.
Jarrett, I. G., and Potter, B. J. (1952): Austral. J. exp. Biol., 30, p. 207.
Long, C. (1951): Biochem. J., 50, p. 407.
Long, C. (1953): *Ibid*, 53, p. 7.
McCandless, E. L., and Dye, J. A. (1950): Amer. J. Physiol., 162, p. 434.
Nelson, N. (1944): J. biol. Chem., 153, p. 375.
Reid, R. L. (1950): Austral. J. agric. Res., 1, p. 182.
Reid, R. L. (1953): *Ibid*, 4, p. 213.
Samuels, L. T., Gilmore, R. C., and Reinecke, R. M. (1948): J. Nutr., 36, p. 639.
Somogyi, M. (1952): J. biol. Chem., 195, p. 19.

EFFECT OF EXTRACELLULAR POTASSIUM ON THE INTRACELLULAR ACTION CURRENT IN THE BEATING HEART IN THE INTACT TOAD[1]

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Much speculation has developed around the mechanism of production of the potential on the surface membrane of the cardiac cell and, indeed, of cells in general. Weidmann (1956) has investigated this problem by measuring the intracellular action current of the turtle heart with changing potassium levels for individual beats initiated by external stimulation, the pace maker having been crushed. We have investigated the problem in the beating toad heart where the change in the intracellular action current contour can be followed sequentially and we have obtained different results, probably because of this last feature.

METHOD.

The Hawaiian toad (*Bufo marinus*) was pithed and a micro-tip inserted into the anterior surface of the exposed heart. A 24 gauge needle was tied into the anterior abdominal vein and this was kept filled with toad Ringer having the electrolyte composition of this species (NaCl 0·65, KCl 0·028, $NaHCO_3$ 0·02, NaH_2PO_4 0·001, Glucose 0·2, $CaCl_2$ 0·012 g.p.c.). Though the toad was pithed, some mouth and upper arm movements were observed occasionally and this probably ensured some oxygen supply to the lungs. Also portion of the visceral surface of the lungs was exposed to the air, and fresh toad Ringer solution was applied to the heart and skin from time to time. Nevertheless, a degree of hypercapnia and anoxia could be expected to develop under these circumstances.

Injections of normal toad Ringer (0·2-0·4 ml.) were made from time to time into the anterior abdominal vein to ensure continuous patency. For the effect of excess potassium, injections were also made of a solution containing 5 milliequivalents per litre potassium in excess of the content of the normal toad Ringer.

Micro-tips, having a resistance of approximately 10 meg Ω, were drawn from 3 mm. glass tubes and filled with 3 M KCl. The drawn tip was 2·5-4 cm. long so that on insertion into the exposed heart it would "splinter" into position and thereby, in favourable instances, the intracellular action current could be measured for some minutes in spite of continuous beating.

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[1] Technical assistance for this work was aided by a grant from the National Health and Medical Research Council, Australia.

EXPERIMENTAL.

The effect of potassium on the intracellular action current of the beating toad heart.

The intracellular action current as recorded in the intact toad heart takes the well-known form as seen in Fig 1(i), (ii). With time in blank trials the horizontal phase of the action current develops a downwards slope as seen in Fig. 2(i) and the contour then remains approximately constant for some minutes and in favourable cases even for hours. The base line, however, slowly rises as is shown in Fig. 2(ii-vi). These changes are referred to here as basic trend.

When potassium is injected intravenously soon after setting up the preparation some of the changes are therefore not due to injected potassium but to this basic trend. If one waits till this basic trend is established the significant effect of potassium is more clearly demonstrated since recovery appears more complete. The basic trend becomes established more rapidly following the injection of potassium i.v. and may itself be due to leakage of potassium from the micro-tip electrode.

(a) *Control injection of toad Ringer.* The intracellular action current is little altered following the intravenous injection of 0·5 ml. of toad Ringer when the tracing becomes stabilized. This is shown in Fig. 2 where between (i) and (ii) 0·4 ml. of normal toad Ringer was injected. Tracing (ii) was obtained "immediately" after injection, i.e. in approximately 5 seconds. Tracings (iii), (iv), and (v), were taken at successive 15 second intervals thereafter. Tracing (vi) was taken 1′ 50″ after injection and tracing (vii) 2′ 50″ after injection.

(b) *Injection of potassium.* Following the injection of 0·4 ml. toad Ringer containing an additional 5 milliequivalents per litre potassium, the descending limb of the complex develops a concavity and the base line rises, the contour then broadens and then temporarily develops a biphasic swing. This is shown in Fig. 2 where the concavity on the descending limb is shown: 2(viii), appearing within 5 seconds of injection. 15 seconds later the descending limb was flattened (ix) and the base line had elevated considerably. At 35 seconds this effect was even more marked (x). At 50 seconds the tracing was similar (xi) and at 1 minute 50 seconds a big biphasic swing reminiscent of the E.C.G. associated with hyper-potassaemia, appeared (xii) though of much greater potential. One minute later a considerable degree of recovery had occurred (xiii) and 1 minute later still (xiv) the descending limb of the complex was very similar to the appearance just before injection of potassium but the complex still showed elevation of the base line and was much broader.

When potassium is injected within a few minutes of setting up the preparation the changes appear more striking (Fig. 1) as referred to above, but some of the changes cannot be attributed to the injected potassium. They may well be due to the leakage of potassium through the micro-tip during the experiment,

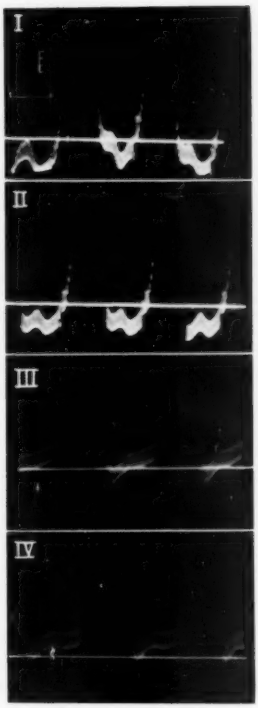

Fig. 1. Toad. Intracellular action current. (i), (ii) before injection of KCl. (iii) 12 sec. after injection of 0·2 ml. toad Ringer containing 8·8 m.equiv K/l. (iv) 35 sec. after injection. Time: 0·6 sec. Potential: 5 mV. Further details in text.

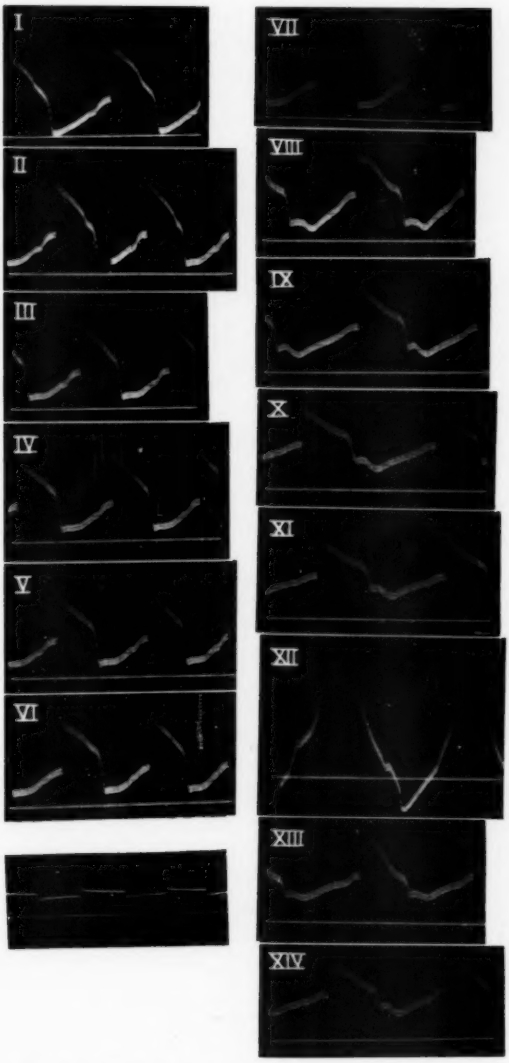

Fig. 2. Toad. Intracellular action current. (i) before injection of normal toad Ringer i.v. (ii) 5 sec. after injection of 0·4 ml. normal toad Ringer. (iii) 20″, (iv) 35″, (v) 50″, (vi) 1′ 50″ after injection. (vii) 2′ 50″ after injection. (viii) 5″ after injection of 0·4 ml. toad Ringer containing 8·8 m.equiv K/l. (ix) 20″, (x) 35″, (xi) 50″, (xii) 1′ 50″, (xiii) 2′ 50″, (xiv) 3′ 50″ after injection. Time: 0·6 sec. Potential: 5 mV. Further details in text.

since an intermittent potential of 100 millivolts is exerted across the $10^7\Omega$ resistance of the electrode. Fig. 1(ii) was taken 30 seconds after Fig. 1(i). Following the injection of 0·2 ml. of toad Ringer containing 5 milliequivalents of potassium per litre in excess of control toad Ringer, elevation of the base line developed with increased slope of the horizontal component of the action current. This is shown in Fig. 1(iii), taken 12 seconds after injection and at 35 seconds (iv) the potential of the complex reduced still further with a concavity appearing on the downward slope. This result was obtained in seven experiments. In other experiments the micro-tip came out of the cell during the manipulations.

DISCUSSION.

If the extracellular potassium is increased the cardiac action potential is reduced in relation to time, potential and rate of rise (Yoshida, 1926), and Weidmann (1956) has shown that when potassium is injected at high concentration (200 mM) giving an estimated solution of 30-80 mM in the perfusate the intracellular action potential showed a more rapid descent after attaining the horizontal phase suggesting quicker repolarization. However, in Weidmann's tracings it is obvious that the return to the base line was much delayed after an initial fall in potential of 70 mv. It is to be remembered that these tracings were obtained in a turtle heart in asystole and the tracing obtained after injection followed one external stimulation of the heart muscle.

In our experiments where the intact beating heart was employed a more prolonged reversal of the potential difference was obtained following the injection of more physiological increments of potassium and this is in agreement with the current theory of Hodgkin (1951). Weidmann suggested three possible alternatives to explain his apparently anomalous findings.

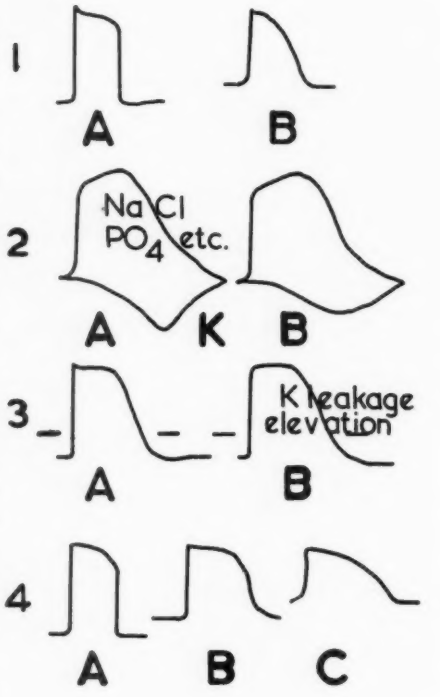

Fig. 3. Diagrammatic representation of intracellular cardiac action current. 1: A. Normal trace. B. Trace some minutes after penetration of cell by micro-tip (N.B. K+). 2: A. Na+ and K+ "migration" components of normal trace. B. Na+ and K+ components after leakage of K+. 3: A. Summation of traces in 2A. B. Summation of traces in 2B. 4: Effect of injected extracellular K+ in addition to injury by micro-tip. A. Normal trace. B. "Basic trend" as in 2B. C. Broadened complex. Further details in text.

Our tracings showed an initial dip in the action potential which was quite minor and overshadowed by the broadening of the action current and the elevation of the base line. Also with greater volumes of injection of Ringer containing excess potassium a large biphasic swing developed in the action potential and this was a reversible result.

It is suggested that our findings are quite consistent with the current theory of Hodgkin (1951). Fig. 3(i) indicates the intracellular action current of the muscle fibre at A; with leakage of potassium around the micro-tip, which must tear the surface especially with the beating heart, the polarization potential, if partly due to potassium normally, must then be reduced, B. The ratio of K^+ outside/K^+ inside is greater than normally and the base line must rise. Similarly the potassium component for action potential will be reduced if the ratio of change of potassium concentration compared to the outside is relatively less. This is shown in Fig. 3(ii) where the normal effect of Na and K migration on potentials is shown at A and the changed response due to increased extracellular potassium from injection of potassium i.v. thereby reducing the ratio to the intracellular K, is shown at B. This will then be associated with a downward slope Fig. 3(iii) B, where ordinarily the action current has a horizontally elevated phase Fig. 3(iii) A. Likewise the introduction of potassium extracellularly might be expected to make the return of potassium delayed and so the action current is broadened as well as having increased slope, C, which can be compared with B and A Fig. 3(iv) showing the sequence of these events. The potential is also reduced in magnitude because of elevation of base line as above. The upper tracing in Fig. 3(ii) A, B indicating Na migration probably encompasses Cl, PO_4 and other ion migration as well as sodium as indicated in the Figure.

SUMMARY.

The effect of injection of K^+ extracellularly on the intracellular action current in the beating toad heart has been investigated.

The significance of these findings was discussed.

Acknowledgment. Grateful thanks are due to Mr. Brian Johnson for stimulating discussions.

REFERENCES.

Hodgkin, A. L. (1951): Biol. Rev., 26, p. 339.
Weidmann, S. (1956): J. Physiol., 132, p. 157.
Yoshida, Von H. (1926): Z. Biol., 84, p. 51.

RESPONSES OF THE PERFUSED FOWL LEG TO POSTERIOR LOBE PITUITARY EXTRACTS

by P. WOOLLEY AND H. WARING

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(*Accepted for publication 16th April, 1958.*)

Strahan and Waring (1954) reviewed previous work, and reported new observations, on the blood pressure responses of the anaesthetised fowl to injections of posterior pituitary extracts.

The anaesthetised fowl responds to injections of whole extract, or pitocin, with a transient fall in blood pressure, and to pitressin by either a rise or fall in pressure according to experimental conditions. Although hitherto there has been no direct evidence from preparations in which the heart is replaced by a pump, it has been generally accepted that the seat of these effects is in the peripheral blood vessels. *Inter alia,* the present paper provides experimental justification for this belief.

The responses to pitocin and pitressin exhibit tachyphylaxis (tolerance) and we (Strahan and Waring, 1954; Waring *et al.*, 1955) have listed possible interpretations of its physiological basis. As a first step to assessing the rôle of several possibilities, Waring *et al.* (1955) performed cross circulation experiments which showed that the tolerant state could be transferred in the blood from one bird to another.

This paper describes experiments on isolated perfused legs which show that tachyphylaxis can be exhibited in the absence both of a heart and of the general circulation.

The experiments are most readily considered under two headings—preliminary and later experiments.

PRELIMINARY EXPERIMENTS.

Materials and Methods.

Animal preparation. Black Australorp roosters of heterogeneous stock and of weights from 1·8 to 4·1 Kg. were anaesthetised with a filtered 10 p.c. aqueous solution of sodium phenobarbitone injected into a wing vein at an average dosage of 200 mg./Kg. The most satisfactory preparations were from birds between 1·8 and 2·2 Kg. All birds were fasted for 24 hours before anaesthesia. It was found preferable to induce anaesthesia slowly over a period of 1 to 2 hours by injection of an initial dose of approximately 50 p.c. of the total dose followed by smaller doses until full anaesthesia was obtained.

Blood for perfusion of the isolated leg was obtained by carotid cannulation of the same donor bird (Parker, 1950) before amputation of the leg. To prevent coagulation heparin

was administered intravenously (1000 i.u./Kg.) 10 minutes before bleeding. An estimated 50 p.c. of the total blood volume was taken and kept in a water bath at 40° C. until required for perfusion.

The feathers were first plucked from the area around the base of the leg and the skin cut to expose the insertions of the leg muscles on the hip bone. The muscles on the dorsal surface were then severed close to the hip bone to avoid cutting branches of the major blood vessels which divide soon after leaving the body cavity. The femoro-sacral joint was dislocated and, after location of the femoral vein and sciatic artery, the remaining muscles, ligaments and the sciatic nerve were severed. The femoral vein and sciatic artery were then clamped with hemostats and severed, the perfusion cannulae were inserted and the pump started, with approximately 10 minutes delay between the arrest of circulation and the start of perfusion. To minimise leakage of blood from the isolated leg, large hemostats were clamped across the cut muscle surfaces. The leg was kept warm on a heated table.

Perfusion technique. In initial experiments an orthodox Dale-Schuster pump was employed. Because of the demands of this pump for a large circulating volume (50 ml.) it was abandoned and a new pump substituted. It utilised the reciprocating mechanism of the Dale-Schuster apparatus, but the pump proper consisted of a wide bore 1 ml. hypodermic syringe attached to a De Vahl-Davis Autovalve. The circulating volume was approximately 20 ml. At full speed and with a stroke volume of 0·15 ml., the output of the pump was 30 ml./minute. Narrow bore non-toxic plastic pressure tubing was used in the perfusion circuit. (Fig. 1).

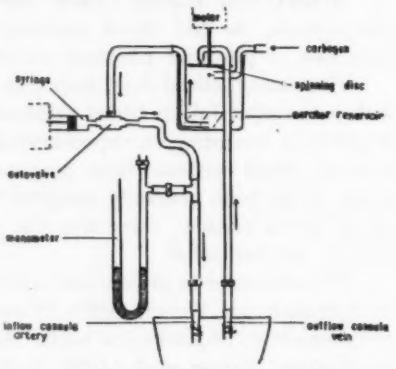

Fig. 1. Perfusion circuit.

The small circulating volume of the pump made it possible to use whole blood as a perfusate. Substitutes for blood investigated in initial experiments, including isotonic saline, Tyrode solution, Tyrode solution plus gum arabic, whole blood diluted 1:8 with Tyrode solution and serum diluted 1:1 with Tyrode solution, had proved unsatisfactory since they led to gross oedema. Aeration of the blood was accomplished by means of a miniature Drinker type aerator through which carbogen (95 p.c. O_2, 5 p.c. CO_2) flowed. Blood was collected by a lead to the pump from the bottom of the aerator reservoir which was contained in a 40° C. water bath.

RESULTS.

In these preliminary experiments perfusion pressures of 3 to 6 cm. of mercury were obtained and only the responses to pitocin were investigated. As in the whole bird, the response to pitocin was a fall in pressure followed by a slower return to basal value. Compared with the whole bird, the preparation was very insensitive. Thus, no measurable response could be elicited by doses smaller than 1 i.u., i.e. about 200 times more than is needed to activate a whole bird. At doses above this, up to about 5 i.u., which evoked a maximal response, there was fair discrimination between doses differing by 1 i.u. Also in contrast to events in the intact bird no decrement in response (tachyphylaxis) to serial injection of pitocin could be demonstrated (Fig. 2, A and B); for example, in

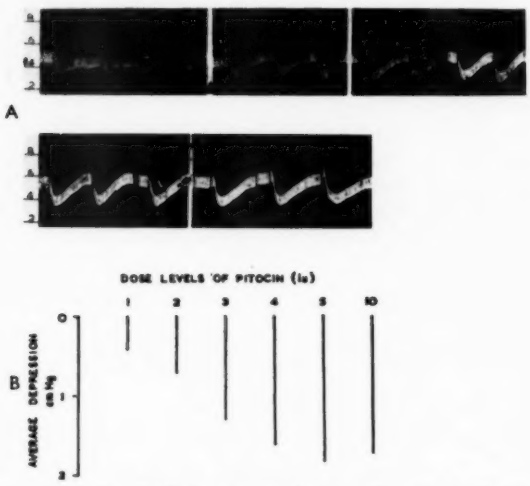

Fig. 2. Preliminary experiments.—Responses of isolated leg to injection of pitocin. (Pressure in cm. Hg.)

A. Injections 6-9 2 i.u.
11-13 3 i.u.
15-19 4 i.u.
20-22 5 i.u.
25-28 10 i.u.

B. Average response to 1 i.u., and each dose level of pitocin in Fig. 2A.

one experiment a dose of 3 i.u. was injected twenty times at 1 to 2 minute intervals and the base pressure and response were unchanged at the end of the series.

While these results suggested that the vascular bed in an isolated leg cannot exhibit tachyphylaxis, the preparation had three obviously unsatisfactory features, low perfusion pressure, seepage of blood from raw surfaces and great insensitivity. The key to making the preparation more sensitive was discovered when we set up similar preparations with the sciatic nerve intact. These experiments are described in the next section.

LATER EXPERIMENTS.

These experiments, with the sciatic nerve intact, arose out of a suggestion that the absence of tachyphylaxis in our earlier preparation was due, in part, to the absence of central nervous connections. In fact, experiments showed that the nerve connection was not essential for exhibition of tachyphylaxis, but the different method needed for setting up a preparation with the nerve intact so improved the sensitivity of the preparation that tachyphylaxis was demonstrated.

METHODS.

No changes were made in the perfusion system. The following modifications were made in preparing the leg. The muscles were carefully dissected away from the hip bone and all minor blood vessels tied and cut as encountered; hemostats were clamped across the cut surfaces of the muscle until an applied hemostatic (Coagulen, Ciba) had sealed the raw surfaces. The femoro-sacral joint was disarticulated to facilitate dissection, but the cartilage surrounding the joint was left intact to hold the leg in position against the body wall, so preventing occlusion of the blood vessels and stretching of the nerve. The leg was now in functional connection with the body by only three structures, the femoral vein, the sciatic artery and the sciatic nerve. Since the sciatic nerve was exposed it could be severed with a single cut, as required, during an experiment. The above procedure was protracted and, as an additional safeguard to the good condition of the preparation, blood for perfusion was not drawn from the same bird but from a separate donor, the compatibility of the two bloods being tested in advance. This procedure also had the advantage that the bird donating the leg needed no heparin, so seepage from the isolated leg was reduced. Bleeding of the donor was carried out after completion of dissection and immediately prior to commencement of perfusion. With this procedure, the time during which blood flow in the leg was arrested, for arterial and venous cannulation, was reduced to 3 minutes. No difficulties were experienced with loss of perfusate during the experiment and perfusion pressures ranging from 5 to 15 cm. Hg were obtained. The net result was a preparation 100 times more sensitive.

RESULTS.

Effect of innervation. The sciatic nerve in the preparation contained functioning vasomotor fibres. This was shown by the observation that pitocin injected into the main circulation of the bird evoked the usual fall in blood pressure while perfusion pressure in the perfused leg rose during the systemic blood pressure trough. There were no such changes in the perfused leg after severing the sciatic nerve.

Injections of pitocin and pitressin into the perfusion circuit produced virtually the same effect on perfusion pressure whether the nerve was intact or severed. In some cases there was a transient fall in pressure as the nerve was cut and in some the base pressure remained slightly lowered, but even in these cases the response to equal doses of pitocin or pitressin administered before or after severing the nerve was of the same magnitude (Fig. 3A).

Response to pitocin. Pitocin evoked the usual depression and the sensitivity was similar to that of whole birds, i.e. a measurable response to doses as small as 0·005 i.u.

The pattern of responses to serial injections varied at different dose levels.

(a) No tachyphylaxis was exhibited to serial injections at maximal speed of small doses of about 0·01 i.u. (Fig. 3A). In the *whole* bird the smallest dose giving a convincing response evoked tachyphylaxis when doses were injected serially at maximal speed; tachyphylaxis can be avoided at these dose levels, as at any other, by suitable spacing of doses.

(b) Rapid serial injections of doses of 0·05 i.u. or higher, elicited responses

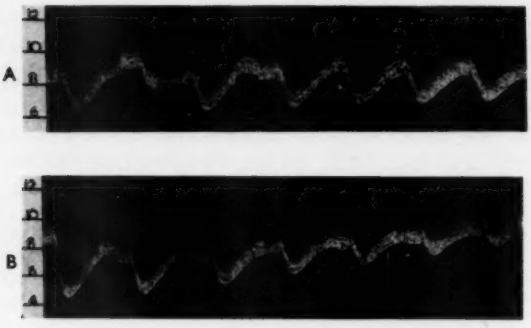

Fig. 3. Responses of isolated leg to serial injections of pitocin. (Pressure in cm. Hg.)

A. Injections 2, and 4 to 8, 0·01 i.u. Nerve severed at 3.

B. Injections 9 to 15, 0·1 i.u.

exhibiting tachyphylaxis (Fig. 3B). As in the intact whole bird, an essential feature of the stabilised tachyphylactic state *at one dose level* was reduction of the depressor response to a residual response for that dose (Waring *et al.*, 1955). This was a greatly diminished response, compared with the original one, but could not be made smaller by further serial injections of the same dose. This sequence of events means that, whatever the mechanism of tachyphylaxis, a stable level of insensitivity, maximal for that dose, is achieved and maintained while injections continue. In the dose range 0·05 to 0·5 i.u. this state is approached gradually with successive doses. Once a residual response for one dose is established, sensitivity can be further reduced by serial doses of greater strength. With a higher dose another stabilised level of insensitivity is eventually achieved as manifested by a residual response to that dose. We have carried this sequence through at three dose levels within the above range. There appeared to be a critical dose (about 0·5 i.u. for most preparations), serial injections of which conferred maximum insensitivity. At, or above, this dose level no further increase of tolerance could be produced. Larger doses gave larger responses, with constant responses to one dose, and after these larger doses the response to the critical dose was not diminished (Fig. 4).

Fig. 4. Isolated leg—maximum tolerance produced by a series of doses.

With doses of 1 to 5 i.u. injected into a fresh preparation there was not the gradual approach to a residual response as for doses of 0·05 to 0·5 i.u. described above; a single injection was usually sufficient to reduce the depressor effect to the residual response.

Information on recovery from the tolerant state, comparable with that from intact birds (Waring *et al.*, 1955) was not obtained in isolated leg experiments because we were unable to keep our preparations in good condition for the requisite period (24 to 72 hours).

Response to pitressin. As with the whole bird, pitressin was less effective than pitocin, the relative potency being about 1:5, which is about the same as in the whole bird. In contrast to the intact bird in which pitressin may have immediate pressor or depressor effects depending on experimental conditions, the effect on the isolated leg perfusion pressure was always an initial fall in pressure followed at times by a secondary rise above basal value. The conditions determining the occurrence of the secondary rise are unknown.

A measurable fall in the perfusion pressure was evoked by pitressin doses from 0·01 to 5·0 i.u.

The responses to serial injections of pitressin followed similar patterns to those obtained for pitocin. No tolerance was developed to serial injections of about 0·04 i.u. or less (Fig. 5A), but progressive decrements in response occurred with serial injections of doses between about 0·1 and 1 i.u. (Fig. 5B). With large doses (over 1 i.u.) maximal tolerance was developed with the first injection. The second injection elicited a greatly diminished fall in pressure, but no further decrement occurred with succeeding injections.

In intact bird preparations the response to injections of pitressin is modified if the preparation is previously made tolerant to pitocin. Under these conditions

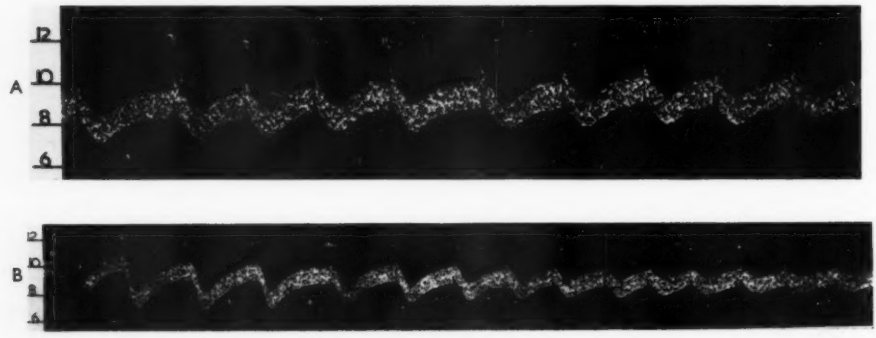

Fig. 5. Responses of isolated leg to serial injections of pitressin. (Pressure in cm. Hg.)
A. Injections 6 to 15, 0·02 i.u.
B. Injections 19 to 33, 0·1 i.u.

the response is always a rise in pressure, regardless of the basal blood pressure (Strahan and Waring, 1954). However, in isolated leg preparations made tolerant to serial 0·1 i.u. doses of pitocin, the response to 0·1 i.u. of pitressin was qualitatively the same before and after tolerance had been induced by pitocin.

Effect of excess magnesium on response to pitressin. Injections of magnesium chloride into intact bird preparations when they are exhibiting a fall to pitressin may augment the fall; when they are exhibiting a rise to pitressin they can, according to other conditions, reduce the rise or convert the expected rise into a fall (Strahan and Waring, 1954). With the isolated leg no such effect was observed. A series of test doses of pitressin, insufficient to induce tolerance, were injected into an isolated leg preparation and then the magnesium content of the perfusate was increased, in some cases up to three times its normal value, with injections of 5 p.c. $MgCl_2$. The injections of $MgCl_2$ alone caused a fall in pressure from which there was never complete recovery. When the blood pressure had stabilised, pitressin injections still evoked depressions.

DISCUSSION.

This paper records observations on the perfused isolated leg of the fowl which have relevance to two responses exhibited by the intact anaesthetised bird: (a) blood pressure changes immediately following injection of posterior pituitary extracts, and (b) tachyphylaxis (tolerance) developed to serial injection. To see how the data from isolated preparations help the analysis of events in the intact animal, it is convenient to comment on these two separately.

(a) *Effect of pitocin and pitressin on fowl blood pressure.* Both pitocin and pitressin diminished inflow pressure in isolated legs perfused at a constant rate. It is clear, therefore, that pituitary extract directly diminishes the resistance to blood flow in muscle and/or skin, presumably by vasodilation. This could explain the immediate drop in blood pressure in the intact bird, invariably by pitocin, and in some circumstances by pitressin. The intact bird responds by a rise to pitressin when basal blood pressure is low; this response we have been unable to reproduce in the isolated leg, but it is an irregular response in the intact bird and is almost certainly (Strahan and Waring, 1954) conditioned not by low blood pressure *per se*, but by an unknown concomitant condition of low blood pressure in some preparations. Pitocin and pitressin reduced the resistance to blood flow through the isolated leg whether the nerve supply was intact or not. In the whole bird this direct effect on the muscle may be integrated by reflex baroreceptor activity, as shown by vasoconstriction in the isolated perfused, but innervated leg, when pitocin was injected into and caused a pressure fall in the systemic circulation.

(b) *Tachyphylaxis.* Experiments with the isolated perfused leg *in situ* show that the heart, splanchnic circulation and nervous connections are not necessarily involved in tachyphylaxis to serial injections of either pitocin or pitressin.

These results also reinforce the conclusion reached earlier, using the intact bird, that the changes mediating tachyphylaxis (a) are virtually immediate in onset, and (b) in response to a given dose are never sufficient totally to extinguish its effect—a residual response always remains. No further light has been thrown on the basic mechanism, but the results are not inconsistent with our earlier suggestion based on cross transfusion that a change in blood constitution is involved (Waring *et al.*, 1955).

The observation that the first injection of a very large dose of either pitocin or pitressin can so reduce the sensitivity of the isolated preparation that only residual responses are exhibited subsequently, explains why our earlier preparations exhibited no tachyphylaxis; they were so insensitive that the first dose needed to activate them put them immediately into the residual response phase. In general, the behaviour of the isolated leg is similar to that of the intact bird, but there are three differences which merit comment.

The observation that the smallest dose that will evoke a depression in the intact bird can also cause a measure of tachyphylaxis, whereas tachyphylaxis does not result from the smallest effective dose in the isolated leg, is consistent with the hypothesis that the insensitivity of tachyphylaxis is due primarily to blood changes. Thus (a) a limited vascular bed and associated tissue (isolated leg) could be expected to yield a smaller maximum of 'insensitivity factor' to the blood than the total bed with a greater potential to be drawn on progressively and (b) if the pituitary-induced insensitivity was vested in the muscle cells *in situ*, as distinct from something carried in the blood, it should be possible to induce the same proportion of total potential insensitivity in small or large amounts of tissue.

There are two responses of the intact bird that we have been unable to reproduce in the isolated leg. The failure to get a rise to pitressin in preparations tolerant to pitocin may be due to this particular response being a complicated one involving differential behaviour of capillary beds in different parts of the whole body: the observation cited in p. 450 would provide an obvious mechanism for the coordination of this. We have no explanation to account for the absence of an effect of increased magnesium content on the response to pitressin in the isolated preparation although it must be recalled that for the clearest demonstration of the effect, a preparation exhibiting elevation to pitressin is necessary, and this we have not achieved.

SUMMARY.

A technique for setting up a perfused isolated fowl's leg is described.

The vasomotor responses of this preparation to posterior lobe pituitary extracts are similar to those of the intact anaesthetised fowl. The preparation exhibits tachyphylaxis to both pitocin and pitressin.

The analysis of the phenomenon of tachyphylaxis in the intact bird is assisted by data from the isolated leg.

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REFERENCES.

Parker, R. C. (1950): Methods of Tissue Culture, 2nd ed., Hoeber, N.Y.
Strahan, R., and Waring, H. (1954): Austral. J. exp. Biol., 32, p. 193.
Waring, H., Crosby, N. D., Woolley, G., Woolley, P., and Marsh, J. (1955): *Ibid.*, 33, p. 103.

THE SIMILARITY OF A RELATIONSHIP BETWEEN BODY WEIGHT AND URINARY 17-KETOSTEROID EXCRETION IN ADULT MALE AND FEMALE HUMAN SUBJECTS

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In their study of the urinary neutral 17-ketosteroid excretion of normal adult male members of six ethnic groups, Lugg and Bowness (1954, 1957a) sometimes encountered, in non-European subjects, levels so far below accepted levels for even female normal European subjects (values of which had been reviewed, for example, by Dorfman, 1948), that they would have been regarded as indicative of pathological conditions had they been found in European males. However, the 17-ketosteroid outputs (K, in mg./24 hr.), or rather some simple logarithmic transformations thereof, were shown to be correlated positively with the creatinine outputs (C, in gm./24 hr.) and with the body-weights (W, in kg.); and evaluation of the expressions, $\log(K+1)/W$, $\log(K+1)/C$ and K/CW (and incidentally C/W) indicated that as far as these compound variates were concerned, the twenty male subjects of each ethnic group might well have been regarded as belonging to the same 'population', whereas for the simple variates, K, C and W, they could not have been so regarded.

It has long been accepted that normal adult female European subjects excrete appreciably less 17-ketosteroids than do their male counterparts (see, for example, Dorfman, 1948), and the difference has rather tacitly been attributed to the lack of testicular contribution in the case of the female (Mason and Engstrom, 1950, for example, emphasize this view). The testicular contribution has somewhat arbitrarily been assessed at 1/3 of the total excretion in males (see, for example, Varley, 1954; Cantarow and Trumper, 1955).

In view of the contents of the first paragraph above, however, and the fact that (as a matter of common observation) female members tend to be smaller in build and lower in weight than the corresponding male members of adult human population groups, it is clear that the difference between male and female levels of 17-ketosteroid excretion merits a careful re-examination. If

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the difference were legitimately to be attributed to the testicular contribution in the case of the male then, perhaps, an appreciable difference between the compositions of the mixture of 17-ketosteroids excreted by the two sexes, might be expected. Yet, no conclusive composition difference has been found (Birke and Plantin, 1954, remark merely that the female may excrete a slightly larger proportion of 11-keto-etiocholanalone). And as between male subjects of various ethnic groups, composition differences are probably very small, if real (Bowness, 1957).

In a survey aimed at assessing less arbitrarily the significance of the male-female differences in 17-ketosteroid excretion, we proposed, in the first instance, to ignore the dubitable composition difference mentioned by Birke and Plantin (1954) and to examine, with those of other variates, values of the compound variates, $\log(K+1)/W$ and $\log(K+1)/C$, encountered among normal adult female members of two dissimilar ethnic groups, comparing these values, both for the two groups themselves and with those of male subjects of the same and other ethnic groups.

In its briefest form the study would thus have involved estimation of the urinary outputs of 17-ketosteroids and creatinine and measurement of the body weight of each subject. The literature concerning creatinine and creatine excretion, however, intimates (1) that whilst the rate of creatinine excretion is a close reflection of the rate of creatinine formation and (in turn) of muscle mass and activity, yet (2) male subjects excrete rather more creatinine for their weight than do female subjects and (3) among normal adult subjects only females are likely to excrete appreciable quantities of creatine. There is no evidence of excessive stimulation in the female of creatine synthesis by substances such as methylated androgens, of endogenous origin, and it seemed advisable therefore to estimate routinely both creatinine and creatine outputs in this study of female subjects, especially in view of earlier suggestions (Shaffer, 1908; Marples and Levine, 1936) that in computing for the female such measures of status as the "creatinine coefficient" (directly related to our C/W), creatinine plus creatine (measured as creatinine) should be regarded as analogous to creatinine for the male.

Among the subjects available to us perhaps the greatest outward pair-dissimilarities (ignoring skin coloration, but involving hard- and soft-tissue morphology and/or body-size) were those apparent between the Negritos and Europeans, the Negritos and Southern Chinese, and the Europeans and Southern Chinese. Of these, only the two last-named types of subject were readily accessible in Singapore, and our study was confined to them.

EXPERIMENTAL.

Subjects. The Chinese subjects were all members of the nursing establishment of the General Hospital, Singapore, and were domiciled in a hostel of that institution. Most of the European subjects were from the nursing establish-

ment of the Royal Air Force Hospital, Changi, Singapore, but several were from the British Military Hospital, Singapore. None of the subjects was known to be pregnant at the time of the urine collection, and none had then entered the climacteric period. Only subjects in the 20-42 year age-range were asked to provide urine samples, and all of them appeared to be physiologically mature and normal. Forbes, Donaldson, Reifenstein and Albright (1947) and Hamblen, Cuyler and Baptist (1941) found no marked variation in the neutral 17-ketosteroid excretion of women in the period following puberty and antecedent to the climacteric, but Hamburger (1948) claimed evidence for a slight drop in excretion between the ages of 25 and 35 years. It would seem that variation with age in the range 20-42 years must be very small, if real.

No information relating the date of urine collection to the menstrual cycle was obtained. From the appearance of the urines, however, no samples seemed to have been supplied during menstruation. Information available in reviews (Dorfman, 1948; Mason and Engstrom, 1950; Dorfman and Shipley, 1956) suggests that there is not normally any considerable fluctuation in the 17-ketosteroid excretion with period in the menstrual cycle.

All subjects lived their normal, everyday lives on Singapore Island, and were thus studied under the same warm-humid climatic conditions as prevailed in our earlier study (Lugg and Bowness, 1957a) of male subjects.

Methods.

Methods for the collection and temporary preservation of the urine were the same as those used in our previous work (Lugg and Bowness, 1957a) except that "polythene" (polymerised ethylene) beakers and funnels were issued with the bottles, and that some of the bottles themselves were of polythene. Urine with added toluene (3 drops) stored in these bottles for 3 days at 0-5° C. was not found to have a 17-ketosteroid content significantly different from the same urine stored in glass bottles under the same conditions of temperature and preservation.

The estimations of the 17-ketosteroids (17-oxo steroids), in terms of dehydroisoandrosterone (dehydroepiandrosterone) as standard, were carried out as described by Lugg and Bowness (1957a). Although the different chromogenic values of the various 17-ketosteroids in the colorimetric method employed impart a degree of arbitrariness to the results, these should (for reasons given earlier) be quite satisfactory for the purposes of comparison intended in the present work.

Weights (including clothing) were measured usually by means of a spring balance, and suitable corrections were applied to bring them to a 'nude' basis.

Estimations of creatinine and of creatinine plus creatine ("total creatinine" calculated as creatinine) were made by the method of Folin (1914): it is of interest to report that the effecting of ring-closure of creatine by autoclaving with hydrochloric acid, prior to treatment with picric acid, was found to hold no advantage over the Folin (1914) procedure.

RESULTS.

Tables 1 and 2 respectively show the basic data for the female Chinese and European subjects, together with means, ranges and estimates of the 'population' standard deviations.

TABLE 1.

Basic data for Chinese female subjects.

Subject	Age in yr.	Urine volume (ml./24 hr.)	Weight in kg. (W)	Creatinine in gm./24 hr. (C)	Creatine plus creatinine in gm./24 hr. (Ct)	17-keto-steroids in mg./24 hr. (K)
C 1	21	2240	54·0	1·23	1·41	8·4
C 2	20	1236	46·8	0·72	0·72	7·3
C 3	20	940	52·1	1·27	1·38	7·7
C 4	21	1635	42·1	0·88	0·88	3·7
C 5	20	945	45·8	1·05	1·14	6·4
C 6	20	915	44·4	0·88	1·03	7·6
C 7	20	1075	47·1	0·82	0·82	4·4
C 8	22	950	45·3	0·82	1·14	6·1
C 9	21	1480	48·1	1·02	1·15	5·5
C10	25	915	42·1	0·70	0·72	6·8
C11	22	1230	44·8	1·08	1·27	9·6
C12	26	1380	42·1	0·81	0·88	4·2
C13	32	1320	53·0	1·06	1·31	4·4
C14	23	780	41·6	1·11	1·16	9·7
C15	33	620	51·6	0·99	1·56	7·5
C16	39	1550	49·0	1·19	1·49	15·0
C17	38	1158	48·6	0·93	1·15	9·4
C18	23	1622	52·6	0·81	1·04	6·7
C19	32	1420	41·2	0·90	1·01	7·0
C20	23	1895	50·3	1·00	1·10	6·5
Mean (M)	25·0	1265	47·13	0·964	1·118	7·20
S.D.	6·20	399·5	4·184	0·1629	0·2422	2·537
Range	Min. 20	620	41·2	0·70	0·72	3·7
	Max. 39	2240	54·0	1·27	1·56	15·0

TABLE 2.

Basic data for European female subjects.

Subject	Age in yr.	Urine volume (ml./24 hr.)	Weight in kg. (W)	Creatinine in gm./24 hr. (C)	Creatine plus creatinine in gm./24 hr. (Ct)	17-keto-steroids in mg./24 hr. (K)
E 1	20	1270	53·9	1·00	1·04	11·3
E 2	21	1820	55·3	1·32	1·38	10·6
E 3	20	1755	49·9	1·16	1·25	8·2
E 4	20	1240	83·9	1·28	1·36	9·6
E 5	26	2830	61·2	1·74	1·90	12·2
E 6	26	1260	59·8	1·13	1·80	10·4
E 7	24	930	70·3	0·85	1·09	10·1
E 8	30	840	70·3	1·13	1·50	8·8
E 9	27	1590	47·1	0·69	1·18	7·0
E10	22	856	45·8	0·95	1·04	10·7
E11	36	1122	56·7	0·90	0·99	5·3
E12	35	1476	68·0	1·31	1·35	11·0
E13	36	1750	51·7	1·06	1·22	4·1
E14	42	1710	49·4	0·79	0·87	3·3
E15	35	1300	57·1	0·90	1·57	10·1
E16	34	520	59·8	0·92	1·10	8·9
E17	36	1945	58·5	1·05	1·15	8·7
E18	25	1730	58·0	1·11	1·21	13·0
E19	27	620	59·0	0·96	1·18	9·1
E20	26	2128	50·3	1·22	1·33	15·4
Mean (M)	28·4	1435	58·30	1·074	1·276	9·40
S.D.	6·64	556·4	9·241	0·2328	0·2609	2·885
Range	Min. 20	520	45·8	0·69	0·87	3·3
	Max. 42	2830	83·9	1·74	1·90	15·4

In addition to the creatinine, total creatinine and 17-ketosteroid outputs and the weights of the subjects, are recorded the ages and the volumes of urine collected.

DISCUSSION.

Interest is centred largely upon W, C, Ct and K, and the distributions of values of these variates are believed to be reasonably representative of those prevailing in the two 'populations' at large, restricted as they are, of course, in respect of health and of chronological and physiological age. In Fig. 1 are shown the within-sample distributions of values of these variates, in the form of frequency polygons constructed, in the manner described by Lugg and Bowness (1957a) and Lugg and Whyte (1955), from the observations distributed (as fractions mostly) between single-valued classes. The centres of the black discs and of the open circles indicate, in each case, the peak and the points of inflection of the fitted 'normal' curve.

There is little in Fig. 1 to suggest that values of any of these variates are other than normally distributed in the parent 'populations'. Attention may be drawn to the somewhat disparate variances in the case of W, the means being relatively little different; and, having regard to the implications of the corresponding values for the male members of these and four other ethnic groups (Lugg and Bowness, 1957a), we are disposed to conclude that in respect of the variate, W, female members of these two ethnic groups, like male members of the six ethnic groups, may not constitute sections of a common 'humanity'. In respect of the variates C, Ct and K, we are obliged to conclude otherwise, but there is a mild distinction between the distributions of our values of K in the present work and those in the work on male subjects in that the results of the present work suggest only a slight positive skewness in the case of the Chinese and do not obviously and promptly impose the desirability of making a logarithmic transformation of K. We have, nonetheless, effected the simple transformation $K \rightarrow \log K$[3] for the purpose of compiling Table 3 and bringing this work into conformity with the work on male subjects. The means (standard deviations in parentheses) of

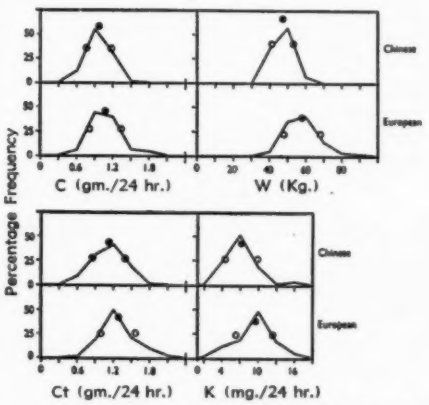

Fig. 1. Frequency distributions of values of C, W, Ct and K in each of the two ethnic group samples.

[3] Throughout, "log X" signifies "logarithm of X to base 10".

values of log K for the female Chinese and European subjects, respectively, are 0·828 (0·142) and 0·948 (0·163).

Table 3 provides tests of significance of the differences between the means of values of the simple variates (and log K) for the female European (M_E ♀) and female Chinese (M_C ♀) subjects, and for these subjects and their male counterparts, M_E ♂ and M_C ♂. Based, as they have been, upon computed values of the "Student" 't' statistic, these tests (strictly speaking), depend for their validity upon normality of the distribution of values of the variate concerned and homogeneity of the variance.

TABLE 3.

Differences between means of the variate values for female European and Chinese, for male and female Chinese, and for male and female European subjects (with computed values for Student's 't' and corresponding probability P of a larger t arising by chance in sampling a single 'population'). The data for male subjects have been taken from Lugg and Bowness (1957a).*

Difference	Variate concerned						
	Age in yr.	Urine volume (ml./24 hr.)	W	C	Ct	K	logK
M_E♀ − M_C♀	3·4	170	11·17	0·110	0·158	2·20	0·120
(t)	1·67	1·11	4·92	1·73	1·98	2·56	2·54
(P)	0·10	0·28	$0{\cdot}0_42$	0·092	0·055	0·015	0·016
M_C♂ − M_C♀	−1·8	298	6·13	0·489	0·335†	2·04	0·112
(t)	1·03	1·69	3·37	6·48	3·92†	2·19	2·39
(P)	0·31	0·10	$0{\cdot}0_216$	$<0{\cdot}0_42$	$0{\cdot}0_337$ †	0·034	0·026
M_E♂ − M_E♀	3·4	105	15·22	0·828	0·626†	6·58	0·238
(t)	1·58	0·63	4·60	9·75	7·04†	5·08	5·21
(P)	0·12	0·53	$0{\cdot}0_47$	$<0{\cdot}0_42$	$<0{\cdot}0_42$†	$0{\cdot}0_42$	$<0{\cdot}0_42$

* Values of P derived, mainly by interpolation, from Pearson and Hartley's (1954) Table 9 (two-tail test) and corresponding with their 2Q values.

† Comparisons of Ct for female with C for male subjects.

Insofar as P is held to represent the chance of two such samples being drawn at random from a common 'population', this interpretation appears to be highly acceptable for log K (but less so for K), and only moderately acceptable for C and Ct and even less so for W, the variances showing appreciable lack of uniformity. That the P values do indicate at least the proper order of the chances in these cases, however, can be shown by employing the 't' statistic of Cochran and Cox (1950) instead of Student's 't'. Of the three sets of pair-comparisons only the first yields a true comparison of Ct values as, in the other two (Ct values for the males being lacking), Ct values for the females have had to be compared with C values for the males; but even so, the differences are much greater than that found in the first comparison. For the most part the differences between the W, C, Ct and K (or log K) values in each pair-comparison are of very considerable statistical significance: the greatest differ-

ences are found in the European male-female comparisons, these being very much greater indeed than those found in the Chinese male-female comparisons.

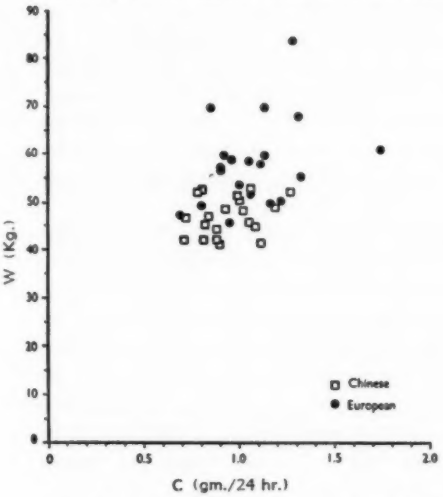

Fig. 2. Relationship between values of W and C.

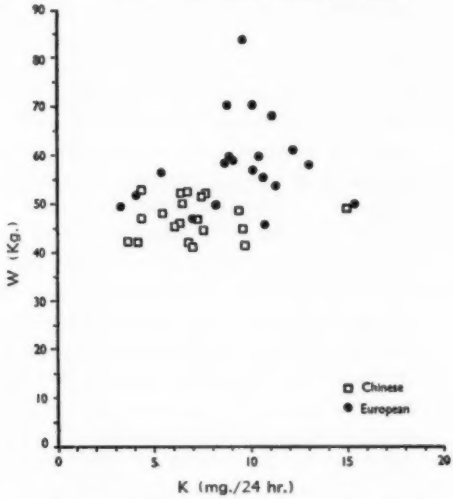

Fig. 3. Relationship between values of W and K.

The 24-hourly urine volumes are deemed to reflect not only the size of the subject but the social and personal fluid-intake habit as well. In this connection it is notable that the European male-female differences are so small despite the considerable differences in weight between the subjects, and we are tempted to attribute the findings to the fact that, of the female subjects in the age-range involved, only the Europeans commonly consume appreciable quantities of alcohol (a diuretic).

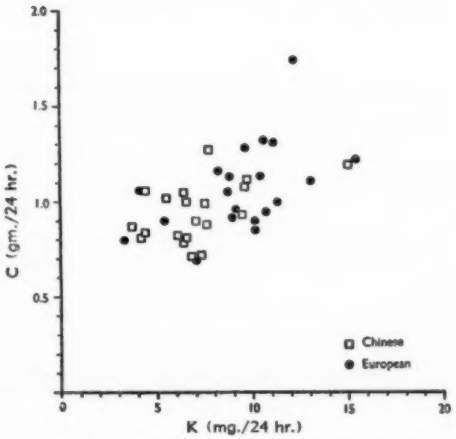

Fig. 4. Relationship between values of C and K.

The age distributions in the samples cannot be held to represent at all adequately the age distributions (within the acceptable age-range) prevailing in the 'populations', but the P values do serve to emphasize the smallness of the inter-sample age-differences in the results we are here discussing.

In Figs. 2, 3 and 4 are shown the relationships (as scatter diagrams) between values of W and C, W and K, and C and K, respectively, for the female subjects.

Attention is drawn to the extreme similarity between the general pattern of each of these diagrams and the pattern of the corresponding diagram for the male subjects (Lugg and Bowness, 1957a), with the implication also that as any two of these three variates must each be expected to tend to zero with the remaining other one, then, as with the data for the male subjects, C might be regarded as varying directly with W, and log K [or better, perhaps, $\log(K+1)$] with W and likewise with C.

Whilst there is relatively little difference between the mean Ct and the mean C values in each case (for the Chinese subjects the former is 116 p.c. of the latter, and for the European, 119 p.c.), yet the creatine outputs exhibit great variability (0-37 p.c. of the Ct output among the Chinese subjects and 3-41 p.c. among the European). The extremely high variability of the creatine outputs does, however, suggest that the excretion many be due to adventitious renal leakage. If this were so, then, inasmuch as there is evidence of a strong positive correlation between the W and C values (see Fig. 2) a diminution in the closeness of correlation might be expected when Ct values replace C values. In Table 4 are shown the correlation coefficients (r) of W with C and of W with Ct, for the Chinese subjects, the European subjects, and all subjects together, and the corresponding probabilities (P) of such correlations arising fortuitously as errors of sampling uncorrelated material.

TABLE 4.

Correlation of W *with* C *and with* Ct, *showing the correlation coefficient* (r) *and the corresponding probability* (P)* *of a larger* r *arising by chance in the absence of any correlation.*

	W with C	W with Ct
Chinese	(r) 0·462 (P) 0·04	(r) 0·600 (P) 0·005
European	(r) 0·339 (P) > 0·1	(r) 0·310 (P) > 0·1
Overall	(r) 0·441 (P) 0·005	(r) 0·471 (P) 0·002

* Values of P derived, mainly by interpolation, from Pearson and Hartley's (1954) Table 13 (two-tail test) and corresponding with their 2Q values.

The Chinese data provide stronger correlations of both W with C and (especially) W with Ct than do the European. However, the overall correlation coefficients of both W with C and W with Ct are of high statistical significance and substantially similar magnitudes: the significance of the difference between the two 'r' values is very low ($\Delta z = 0{\cdot}037$, whence 't' for ∞ d.f. $= 0{\cdot}11$, and $P > 0{\cdot}8$). Clearly, the expectation based upon the hypothesis referred to

in the previous paragraph has not been realized, and the analysis of the data thus far fails to indicate clearly whether C or Ct with female subjects might be viewed as the more strict analogue of C with male subjects.

In Table 5 are shown, for the two female groups, separately and overall, the means of values of the compound variates, C/W, Ct/W, log(K + 1)/W, log(K + 1)/C, log(K + 1)/Ct, and K/CW and K/CtW, together with the

TABLE 5.

The means, standard deviations and ranges of values of compound variates for the Chinese and for the European female subjects and for them all together.

Subjects and statistics		$\frac{C}{W}$	$\frac{Ct}{W}$	$\frac{\log(K+1)}{W}$	$\frac{\log(K+1)}{C}$	$\frac{\log(K+1)}{Ct}$	$\frac{K}{CW}$	$\frac{K}{CtW}$
Chinese	Mean	0·0205	0·0236	0·0190	0·942	0·825	0·160	0·138
	S.D.	$0{\cdot}0_3305$	$0{\cdot}0_3423$	$0{\cdot}0_3303$	0·167	0·180	0·0496	0·0463
Range	Min.	0·0154	0·0154	0·0138	0·691	0·559	0·078	0·063
	Max.	0·0267	0·0304	0·0247	1·276	1·276	0·258	0·223
European	Mean	0·0186	0·0221	0·0174	0·955	0·800	0·155	0·130
	S.D.	$0{\cdot}0_3398$	$0{\cdot}0_3445$	$0{\cdot}0_3324$	0·180	0·121	0·0523	0·0471
Range	Min.	0·0121	0·0155	0·0122	0·646	0·580	0·075	0·066
	Max.	0·0284	0·0310	0·0242	1·309	1·048	0·251	0·230
Overall	Mean	0·0196	0·0228	0·0182	0·949	0·812	0·158	0·134
	S.D.	$0{\cdot}0_3355$	$0{\cdot}0_3434$	$0{\cdot}0_3314$	0·174	0·153	0·0510	0·0467
Range	Min.	0·0121	0·0154	0·0122	0·646	0·559	0·075	0·063
	Max.	0·0284	0·0310	0·0247	1·309	1·276	0·258	0·230

ranges and estimates of the 'population' standard deviations; and the 'overall' statistics have been derived on the assumed homogeneity of the individual group means and variances. In Fig. 5 are shown the within-sample distributions of values of the first five of these compound variates, the frequency polygons being constructed in a way entirely analogous to that which yielded Fig. 1, and the black discs and open circles again respectively indicating the peaks and points of inflexion of the fitted 'normal' curves. No definite de-

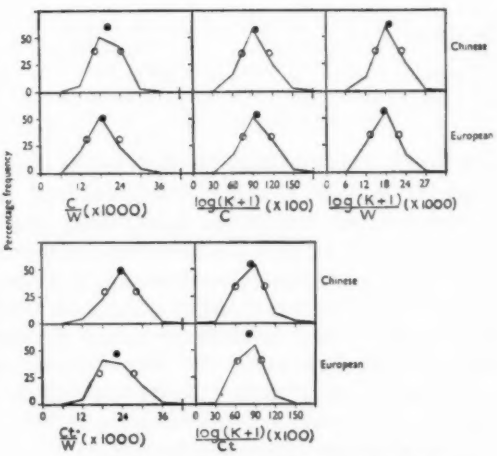

Fig. 5. Frequency distributions of values of some compound variates in each of the two ethnic group samples.

partures of the population distributions from 'normality' are apparent in Fig. 5, and the inter-group differences are clearly quite small. From Table 5 it would seem that the final two compound variates (as well as the first five) have very similar values for the two female groups.

Such disparities as may exist between the two groups of female subjects have been examined (see Table 6) as the differences between the means (M_E ♀

TABLE 6.

Differences between means (and tests of the statistical significance thereof:—computed values of Student's t *and values of the probability,* P, *derived as indicated in a footnote to Table* 3) *of the compound variate values for Chinese and European female subjects, for these and their male counterparts, and for other associations of subjects, the data for male subjects being those of Lugg and Bowness* (1957a).

Differences	$\frac{C}{W}$	$\frac{Ct}{W}$	$\frac{\log(K+1)}{W}$	$\frac{\log(K+1)}{C}$	$\frac{\log(K+1)}{Ct}$	$\frac{K}{CW}$	$\frac{K}{CtW}$
M_E♀-M_C♀	$-0{\cdot}0_319$	$-0{\cdot}0_315$	$-0{\cdot}0_316$	0·013	−0·025	$-0{\cdot}0_35$	$-0{\cdot}0_38$
(t)	1·69	1·09	1·56	0·24	0·52	0·31	0·54
(P)	0·098	0·28	0·13	0·81	0·60	0·76	0·59
M_C♂-M_C♀	$0{\cdot}0_270$	$0{\cdot}0_239$†	$-0{\cdot}0_31$	−0·239	−0·122†	−0·037	−0·015†
(t)	5·03	2·53†	0·10	4·78	2·34†	2·61	1·10†
(P)	$0{\cdot}0_42$	0·016†	0·92	$0{\cdot}0_48$	0·024†	0·013	0·28†
M_E♂-M_E♀	$0{\cdot}0_375$	$0{\cdot}0_340$†	$-0{\cdot}0_36$	−0·304	−0·149†	−0·036	−0·011†
(t)	6·47	3·21†	0·58	6·49	4·12†	2·44	0·84†
(P)	$<0{\cdot}0_42$	$0{\cdot}0_327$†	0·56	$<0{\cdot}0_42$	$0{\cdot}0_33$†	0·020	0·41†
M_{CE}♂-M_{CE}♀	$0{\cdot}0_272$	$0{\cdot}0_340$†	$-0{\cdot}0_34$	−0·272	−0·135†	−0·037	−0·013†
(t)	5·62	2·86†	0·41	5·58	3·00†	2·55	0·94†
(P)	$<0{\cdot}0_42$	$0{\cdot}0_355$†	0·68	$<0{\cdot}0_42$	$0{\cdot}0_335$†	0·013	0·15†
M_{CEIMNS}♂-M_{CE}♀	$0{\cdot}0_260$	$0{\cdot}0_328$†	$-0{\cdot}0_38$	−0·249	−0·112†	−0·046	−0·022†
(t)	6·26	2·84†	1·36	8·69	4·05†	5·96	2·94†
(P)	$<0{\cdot}0_42$	$0{\cdot}0_352$†	0·17	$<0{\cdot}0_42$	$0{\cdot}0_48$†	$<0{\cdot}0_42$	$0{\cdot}0_338$†

† Comparisons involving Ct for female subjects with C for male subjects.

and M_C ♀), the statistical significances of these being shown by the calculated values of Student's 't' and the magnitudes of the corresponding probabilities, P. The warrant for pooling the female data (see Table 5) as a means of arriving at a set of values likely to be more representative of human female values at large in the age-range involved here, is confirmed by the fairly large magnitudes of P for the M_E ♀-M_C ♀ values in Table 6. Table 6 shows also the differences between the means (and the statistical significances of the differences) of the composite variate values for the Chinese male (M_C ♂) and female subjects, the European male (M_E ♂) and female subjects, the combined Chinese and European male subjects (M_{CE} ♂) on the one hand and the corresponding combined female subjects (M_{CE} ♀) on the other, and finally, the combined Chinese, European, Indian, Malay, Negrito and Sēnoi male subjects (M_{CEIMNS} ♂) on the one hand and the combined Chinese and European female subjects on the other.

Brief reference has already been made (Lugg and Bowness, 1957b) to one of the most important conclusions to be drawn from Tables 5 and 6 and Fig. 5, namely, that of all the compound variates under consideration here, $\log(K+1)/W$ alone appears to have closely similar values for male and female subjects. This slightly tentative conclusion was actually based upon consideration of the M_{CEIMNS} ♂ -M_{CE} ♀ differences only; but the more detailed information provided in Table 6 allows us to repose much confidence in the conclusion as a generality, because it emerges clearly from each of the four male-female comparisons shown there. By the same token, the conclusion that the other variates have more-or-less dissimilar values for male and female subjects is strongly reinforced by the more detailed information in Table 6; and it is of great interest to observe that for any particular one of these variates the differences are of similar magnitude for all four of the male-female comparisons. In passing, however, it might be indicated that the information in Table 5 does lend some support to a suspicion that slight apparent racial differences, of the order encountered by Lugg and Bowness (1957a) with male subjects, may not be entirely due to random errors of sampling.

In a general way the differences between male and female values of compound variates involving C are reduced if Ct replaces C for the female subjects, but they are certainly not changed in sign and are by no means obliterated, though in the case of Ct/W they are of much diminished significance. Thus, these results fail to support the view of Shaffer (1908) and of Marples and Levine (1936), but, contrariwise, sustain the objection of Catherwood and Stearns (1937) to this view.

The finding that values of $\log(K+1)/W$ are virtually unaffected by the sex difference whereas those of C/W and of $\log(K+1)/C$ are markedly affected thereby, clearly stands in conflict with the commonly-implied relationship (for example, see Mason and Engstrom, 1950, and other references given in the introduction) between the rate of excretion of neutral 17-ketosteroids and sexuality in human subjects—a conclusion presaged by the results of our earlier work with male subjects alone (Lugg and Bowness, 1957a).

It is known that androgens are produced in the adrenal gland and in the testis, and that they are probably produced in the ovary, and possibly in the placenta (see the reviews of Dorfman, 1948; Parkes, 1950; Deane and Seligman, 1953). Little is known, however, about the relative amounts of androgenic material actually secreted by the four tissues, the levels at which they and their metabolic products circulate in the blood and the relationships between these levels and the rates of excretion of the various 17-ketosteroids (the observations of Migeon, 1956, and of Kellie and Smith, 1957, for example, are conflicting); but if it be assumed (as a first approximation, and neglecting placental contributions) that the relationships are linear and similar in the two sexes then, from the outcome of the present work and the observations of Birke and Plantin

(1954) concerning the compositions of excreted 17-ketosteroids, it seems that the androgens secreted by the two sexes may be rather similar in composition and (with due regard to body-weight) similar in amount.

In respect of oestrogen production it is known that that of the male subject is by no means insignificant—indeed, as far as the testicular hormonal secretion itself is concerned, Berthrong, Goodwin and Scott (1949) regarded the oestrogen as second only to the androgen component. Yet, a feature both of production levels (insofar as these may be adequately indicated by urinary levels of excretion products) and of circulating blood levels (from direct assays of serum) in female subjects of the physiological status involved in our work, is their dependence upon the stage of the menstrual cycle, the urinary levels in the mid-part of the cycle rising far above the levels commonly encountered in male subjects and apparently being related throughout the cycle to the urinary levels of progestational hormone excretory products (for a general review see Cantarow and Schepartz, 1957).

It is apparent from our own work that in human subjects sexuality must be linked much more closely with the creatinine (and, perhaps, creatine) than with the neutral 17-ketosteroid excretion; and in the light of the foregoing discussion we incline to the view that in considerations of the sex hormones as factors in the maintenance of normal sex differences attention should, perhaps, be directed towards the gynaecogens rather than the androgens.

SUMMARY.

A study of the relationships in twenty each of normal pre-menopausal adult, non-pregnant, female, Southern Chinese and European subjects, between the weights (W, in kg.), urinary 17-ketosteroid excretion (K, in mg./24 hr.), urinary excretion of creatinine (C, in gm./24 hr.) and of creatinine plus creatine calculated as creatinine (Ct, in gm./24 hr.), and comparison of the results with those obtained by Lugg and Bowness (1957a) with normal adult male subjects belonging to a variety of ethnic groups, has led to the following conclusions:

Distributions of values of $\log(K+1)/W$ are similar for both female and male subjects of normal adult human groups of varying ethnic derivation, although distributions of values of W and K (and of C) may vary greatly with the sex and from group to group.

Values of $\log(K+1)/C$ and also of K/CW appear to be very similar for the normal adult female members of different ethnic groups, but are appreciably larger than those for male members, values of C/W being smaller.

Values of $\log(K+1)/Ct$ and also of K/CtW, probably similar for normal adult female members, and smaller in general than the corresponding $\log(K+1)/C$ and K/CW values, appear still to be appreciably larger than the values of these latter variates for male subjects, values of even Ct/W for female subjects being smaller than those of C/W for male.

Thus, contrary to a commonly accepted view, the association in normal adult human subjects between sexuality and neutral 17-ketosteroid excretion appears to be no closer than that between sexuality and body-weight.

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REFERENCES.

Berthrong, M., Goodwin, W. E., and Scott, W. W. (1949): J. clin. Endocrin., 9, p. 579.
Birke, G., and Plantin, L.-O. (1954): Acta Med. Scand., 148 (Suppl. 291), p. 7.
Bowness, J. M. (1957): In Lugg and Bowness (1957a): Appendix.
Cantarow, A., and Schepartz, B. (1957): "Biochemistry", 2nd Edit., Saunders, Philadelphia, U.S.A.
Cantarow, A., and Trumper, M. (1955): "Clinical Biochemistry", 5th Edit., Saunders, Philadelphia, U.S.A.
Catherwood, R., and Stearns, G. (1937): J. biol. Chem., 119, p. 201.
Cochran, W. G., and Cox, G. M. (1950): "Experimental Designs", John Wiley & Sons Inc., New York, U.S.A.
Deane, H. W., and Seligman, A. M. (1953): Vit. and Horm., 11, p. 173.
Dorfman, R. I. (1948): In "The Hormones", Ed. Pincus, G., and Thimann, K. V., Academic Press, New York, U.S.A.
Dorfman, R. I., and Shipley, R. A. (1956): "Androgens", John Wiley & Sons Inc., New York, U.S.A.
Folin, O. (1914): J. biol. Chem., 17, p. 469.
Forbes, A. P., Donaldson, E. C., Reifenstein, E. C., Jr., and Albright, F. (1947): J. clin. Endocrin., 7, p. 264.
Hamblen, E. C., Cuyler, W. K., and Baptist, M. (1941): *Ibid.*, 1, p. 777.
Hamburger, C. (1948): Acta Endocrin., 1, p. 19.
Kellie, A. E., and Smith, E. R. (1957): Biochem. J., 66, p. 490.
Lugg, J. W. H., and Bowness, J. M. (1954): Nature, Lond., 174, p. 1147.
Lugg, J. W. H., and Bowness, J. M. (1957a): Austral. J. exp. Biol., 35, p. 395.
Lugg, J. W. H., and Bowness, J. M. (1957b): Austral. J. Sci., 20, p. 54.
Lugg, J. W. H., and Whyte, J. M. (1955): Ann. hum. Genet, Lond., 19, p. 290.
Marples, E., and Levine, S. Z. (1936): Amer. J. Dis. Child., 51, p. 30.
Mason, H. L., and Engstrom, W. W. (1950): Physiol. Rev., 30, p. 321.
Migeon, C. J. (1956): J. biol. Chem., 218, p. 941.
Parkes, A. S. (1950): Recent Prog. Horm. Res., 5, p. 101.
Pearson, E. S., and Hartley, H. O. (1954): "Biometrica Tables for Statisticians, Vol. 1", Cambridge Univ. Press, Eng.
Shaffer, P. (1908): Amer. J. Physiol., 23, p. 1.
Varley, H. (1954): "Practical Clinical Biochemistry", Heinemann, London, Eng.

THE EFFECTS OF HEAVY METAL IONS ON THE HUMAN ERYTHROCYTE

I. COMPARISONS OF THE ACTION OF SEVERAL HEAVY METALS

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The effect of heavy metal ions on the permeability of the red cell has received considerable attention and, in particular, the loss of intracellular potassium resulting from the action of Hg, Pb, Au on the rabbit erythrocyte (Joyce *et al.*, 1954) and from the action of Pb on the human red cell (Ørskov, 1935; Henriques and Ørskov, 1936; Passow and Tillmann, 1956). Changes in the membrane of red cells treated with Pb have been indicated by findings of an increased osmotic resistance and mechanical fragility (Aub *et al.*, 1925), by agglutination of poisoned cells (Jandl and Simmons, 1957), and by the appearance of a direct positive Coombs test in lead poisoning (Sutherland and Eisentraut, 1956). Recently the reduction in the erythrocyte sedimentation rate (ESR) of human red cells, brought about by certain hyaluronidase preparations, was shown to be due to the lead content of these preparations (Blackburn, 1949). Concomitant findings were a decrease in cell volume and a loss of cell potassium.

The present paper reports studies of the effects of Pb++, Hg++, Au+++, Cu++, Cd++ and Zn++ salts, and of certain other substances, on the potassium metabolism of human red cells.

METHODS.

Packed normal human erythrocytes were separated from freshly collected heparinized whole blood by centrifugation at 3000 r.p.m. for fifteen minutes. They were washed twice with cold isotonic sodium chloride or sodium acetate and suspended in an equal volume of 0·025M glucose made isotonic with the sodium analogue of the heavy metal salt under study.

Incubation of a known volume of normal human red blood cells with an appropriate amount and concentration of the heavy metal salt was carried out at 37° C. for 60 minutes with gentle agitation, after which the cells were separated by centrifugation at 3000 r.p.m. for five minutes and the supernatant aspirated. The supernatant so collected was assayed

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by flame photometry for potassium, using at first a lithium internal standard type flame photometer,[2] and later an "EEL" flame photometer.

The intracellular potassium and haemoglobin concentrations of each batch of cells were determined after the lysis of 0·1 ml. of the cell suspension in 20 ml. of 1/400 ammonium hydroxide. The potassium was estimated as described above and the haemoglobin by the method of Heilmeyer (1943) in which extinction coefficients at wavelengths of 541 and 560 mμ were read in a Beckman model DU spectrophotometer. Haematocrit measurements were made in large (6 ml.) tubes, spun at 3000 r.p.m. for 30 minutes at a radius of 15 cm.

EXPERIMENTAL.

The addition of not more than 0·67 μmoles of $ZnCl_2$, $CdCl_2.2H_2O$, $CuCl_2$, $HgCl_2$, $Hg(NO_3)_2$, $AuCl_3HCl.2H_2O$, $Pb(CH_3COO)_2$ or $Pb(NO_3)_2$ per ml. of red cells resulted in K^+ loss from these cells, but the addition of up to 2 μmoles of $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, $MnCl_2.4H_2O$ and $FeCl_3.6H_2O$ per ml. of cells did not have this effect.

Some haemolysis occurred when 2 μmoles of $FeCl_3.6H_2O$ were added to 1 ml. of red cells, and the K^+ loss was quantitatively related to this cell destruction. No haemolysis was observed with $CoCl_2.6H_2O$, $NiCl_2.6H_2O$ or $MnCl_2.6H_2O$.

Direct comparisons of the activity of Pb^{++}, Hg^{++}, Au^{+++}, Cu^{++}, Cd^{++} and Zn^{++} in respect of their effect on the K^+ metabolism of the red cells were made and the results are set out in Table 1 and Fig. 1.

It can be seen that the metals have differing abilities to release K^+ from the cells and whether the K^+ released is expressed as μEq/ml. of cells or as a ratio in relation to the μmoles of metal added, the metals fall into an order of effectiveness of $Pb^{++} > Hg^{++} > Au^{+++} > Cu^{++} > Cd^{++} > Zn^{++}$ as shown in Fig. 1. A molar scale is used since we have no evidence that the electrostatic charges determine the activity of the metal ions. Comparison of the anions of the different metal salts showed that quantitative, but not qualitative, differences

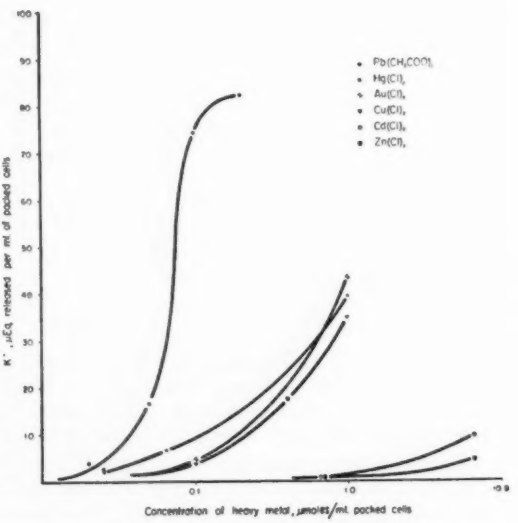

Fig. 1. The effects of Pb, Hg, Au, Cu, Cd and Zn ions on the loss of K from the red cell after incubation at 37° C. for 60 minutes.

[2] Lent by Dr. Gilbert H. Mudge.

TABLE 1.

K^+ released by the action of metals on normal human red cells. Temperature 37° C, time of exposure 60 minutes, cell volume 0·25 ml., supernatant volume 4·75 ml. K^+ measured in supernatant.

Metallic Salt	Metal added per ml. of cells μmoles	Haemolysis	K^+ released μEq/ml cells μEq	K^+ released μEq / Metal added μmoles
$Pb(CH_3COO)_2$	0·20	—	82·46	412
	0·10	—	74·93	749
	0·05	—	10·64	213
	0·025	—	2·97	119
	0·020	—	4·15	207
	0·002	—	0	0
$Pb(NO_3)_2$	0·10	—	80·56	806
	0·01	—	31·78	3180
	0·001	—	1·32	1320
	0·0001	—	0	0
$HgCl_2$	1·00	—	39·36	39
	0·40	—	17·36	43
	0·16	—	14·96	93
	0·064	—	6·52	102
	0·025	—	2·53	101
	0·010	—	0	0
$Hg(NO_3)_2$	1·00	—	42·72	43
	0·10	—	41·96	420
	0·01	—	3·14	314
	0·001	—	0	0
$AuCl_3$	10·0	—	50·0	5
	1·0	—	43·2	43
	0·1	—	4·2	42
	0·01	—	0	0
$CuCl_2$	1·0	—	34·52	34
	0·1	—	4·28	43
	0·01	—	0	0
$CdCl_2$	66·0	—	43·7	0·7
	6·6	—	9·3	1·4
	0·66	—	0·29	0·4
	0·066	—	0	0
$ZnCl_2$	6·6	—	4·5	0·7
	0·66	—	0·24	0·4
	0·066	—	0	0

could easily be due to differences in dissociation constants, for example, gold thiomalate was without effect on the cells.

The order of activity of the metals, expressed as the ratio of μEq K^+ released to μmoles heavy metal added, at the level of 0·1 μmoles/ml. of cells is 750-800 for Pb, 100-400 for Hg, 40 for Au and Cu, 0·5 for Cu and Zn.

All the "active" metals are inhibitors of thiol dependent enzymes (e.g., succinoxidase) presumably by the formation of relatively undissociated mer-

captides. For this reason, certain other thiol group inhibitors were studied, but sodium arsenate, sodium arsenite, sodium iodoacetate and sodium tetrathionate were all ineffective in concentrations greater than those found effective for the metal salts.

The release of K^+ from red cells was dependent, not on the concentrations of the heavy metal in the suspending medium, but on the ratio of μmoles heavy metal to units of red cells. This was demonstrated by exposure of cell suspensions of varying concentrations to varying concentrations of heavy metal, the two concentrations being related so that the ratio of total metal to total cells remained constant. Table 2 shows the results of an experiment in which cell suspensions, varying between 5 and 25 p.c., were exposed to concentrations of Hg calculated to maintain a constant ratio between total Hg and the cells of 0·4 μmoles/ml. packed cell volume. In these conditions the *concentration* of Hg exposed to the red cell membrane varied from 0·02 μmoles/ml. to 0·10 μmoles/ml. In Fig. 2, the comparison is extended to several molar Hg/packed cell ratios. It will be seen that in all cases studied the K^+ lost from the red cell is related,

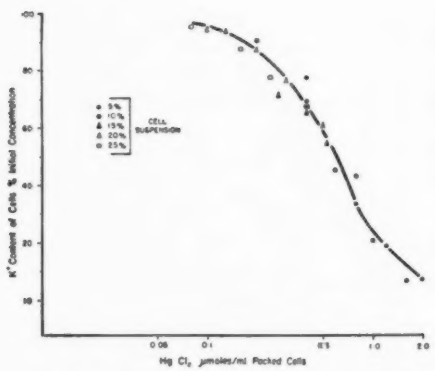

Fig. 2. The expression of Hg concentration with respect to the volume of packed red cells. The loss of K from red cells is related to the molar ratio of Hg per ml. packed cells. Since this is true for different concentrations of cell suspension, it follows that the K efflux is not directly related to the supernatant concentration of Hg.

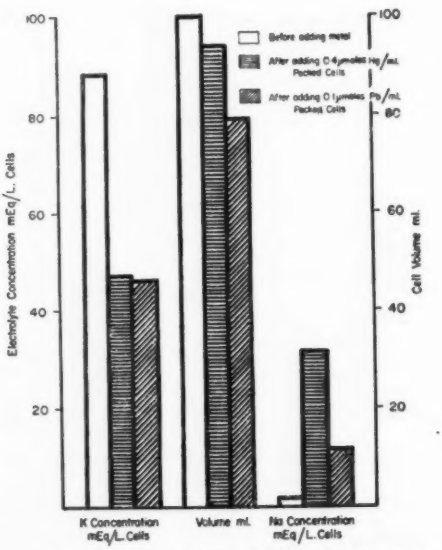

Fig. 3. The effects of Pb and Hg on the red cell. This figure illustrates the greater decrease in volume, and the smaller Na^+ uptake, of cells treated with Pb compared with those treated with sufficient Hg to cause a similar K^+ loss.

TABLE 2.

The loss of K^+ from a system in which the ratio of added Hg^{++} to red cells is constant whilst the concentration of Hg^{++} in the suspending medium is varied. Suspensions of normal human red cells (5% to 25%) were incubated in the presence of $HgCl_2$ (0·02 to 0·10 μmoles/ml.) for 60 minutes at 37° C. Ratio Hg^{++}/cells 0·4 μmoles/ml. of packed cells and total volume 5·0 ml.

Cell suspension %	Hg concentration μmoles/ml	Hg expressed as ratio $\frac{\mu\text{moles } Hg^{++}}{\text{ml. packed cells}}$	K^+ released μEq. ml. packed cells
5	0·02	0·4	31·9
10	0·04	0·4	27·9
15	0·06	0·4	29·4
20	0·08	0·4	26·6
25	0·10	0·4	24·5

TABLE 3.

Comparison of cellular and supernatant volumes, Na^+ and K^+ of systems in which Hg or Pb were incubated with normal human red cells in isotonic NaCl for one hour at 37° C.

(The volume readings have been corrected for trapped plasma volume).

Metal	Part of system studied		(1) Control	(2) With metal	(3) Difference Actual	(3) Difference As p.c. original
Hg 0·4 μmoles per ml. packed cells	Cells	Volume, ml.	1000	944	− 56	−5·6
		Total K^+, mEq.	88·5	44·7	− 43·8	−49·5
		K^+, mEq/L	88·5	47·5	− 41·0	−46·4
		Total Na^+, mEq.	1·6	30·0	+ 28·4	+1780
		Na^+, mEq/L	1·6	31·8	+ 30·2	1900
		$Na^+ + K^+$, mEq.	90·1	74·7	− 15·4	−17·1
		$Na^+ + K^+$, mEq/L	90·1	79·3	− 10·8	−12·0
	Supernatant	Volume, ml.	2610	2680	+ 70	+2·7
		Total K^+, mEq.	0	39·4	+ 39·4 } theoretically	
		K^+, mEq/L	0	15·3	+ 15·3 } infinite	
		Total Na^+, mEq.	440·0	406·0	− 34·0	−8·2
		Na^+, mEq/L	168·0	152·0	− 16·0	−9·5
		$Na^+ + K^+$, mEq.	440·0	445·4	+ 5·4	+1·2
		$Na^+ + K^+$, mEq/L	168·0	167·3	− 0·7	−0·4
Pb 0·1 μmoles per ml. packed cells	Cells	Volume ml.	1000	795	−205	−20·5
		Total K^+, mEq.	80·5	33·2	− 47·3	−58·6
		K^+, mEq/L	80·5	42·0	− 38·5	−47·8
		Total Na, mEq.	2·9	10·6	+ 7·7	+266
		Na^+, mEq/L	2·9	13·3	+ 10·4	+360
		$Na^+ + K^+$, mEq.	83·4	43·8	− 39·6	−47·5
		$Na^+ + K^+$, mEq/L	83·4	55·3	− 28·1	−33·7
	Supernatant	Volume ml.	1400	1595	+195	+14·0
		Total K^+, mEq.	4·2	59·0	+ 54·8	+1300
		K^+, mEq/L	3·0	37·0	+ 34·0	+1133
		Total Na, mEq.	211·0	197·0	− 14·0	−6·7
		Na^+, mEq/L	151·0	124·0	− 27·0	−18·0
		$Na^+ + K^+$, mEq.	215·2	256·0	+ 40·8	+19·0
		$Na^+ + K^+$, mEq/L	154·0	161·0	+ 7·0	+4·5

not to the concentration of mercury present in the supernatant, but to the concentration per ml. packed cells.

The results show clearly that, under these conditions, there was no significant difference between the K^+ loss following the exposure of red cells to concentrations of Hg differing as much as five-fold from each other. Therefore, the expression of the amount of metal added as a concentration, unless the total volume and the volume of cells used are comparable in all cases, will lead to fallacious results and may explain differences in published results of different authors. We have expressed the amounts of heavy metal added to red cells throughout these studies as a ratio of μmoles of metal/ml. packed cell volume, a measure which can be conveniently described as the "dose" of heavy metal added to each unit of cells.

Pb and Hg were found to differ in their effects on red cell permeability to K^+ and Na^+ (Table 3 and Fig. 3). If either metal was allowed to act on red cells in a NaCl environment there was a rapid loss of intracellular K^+; but a significant and early coincident gain in cell Na^+ was only observed after exposure to Hg and this with little change in cell volume. Immediately after the addition of Pb, on the other hand, little Na^+ passed into the cell in spite of a marked K^+ loss and this was accompanied by a pronounced diminution in cell volume.

Although the loss of intracellular K^+ was slightly greater under the action of Pb than of Hg the difference was not great enough to explain the difference in changes in cell volume, intracellular $(Na + K)$ concentration, or the total $(Na + K)$ in the cells between the two metals. This is shown by abstraction of data from Table 3 thus:

	Pb	*Hg*
Change in cell volume - -	− 21 p.c.	− 6 p.c.
Change in total Na + K - -	− 48 p.c.	− 18 p.c.
Change in Na + K concn. -	− 34 p.c.	− 13 p.c.
Change in total intracellular K	− 59 p.c.	− 50 p.c.

These differences may be attributed, in the main, to a greater flow of water and Na^+ into the cells exposed to Hg than to Pb, but obvious discrepancies in Table 3 suggest that some other factor is active, especially in the case of Hg, to retain water inside the cell.

The relation between μmoles of metal added to the cells and μEq K released was different for the two metals since the ratio $\frac{\mu\text{Eq K released}}{\mu\text{moles metal added}}$ reached 100 for Hg but for Pb it reached 1,000.

DISCUSSION.

Of the metals studied, only Pb^{++}, Hg^{++}, Au^{+++}, Cd^{++}, Cu^{++} and Zn^{++} were found to be active in promoting the rapid loss of K^+ from normal human

red cells. Although these metals were also the only ones capable of ready combination with thiol groups, typical non-metallic thiol inhibitors were without effect on releasing K^+.

An ionic series of activity for six of the heavy metals was established and metals promoted K^+ loss from the red cell in the following order: $Pb > Hg > Au > Cu > Cd > Zn$, which is the reverse of the order of activity of the same metals in inhibition of the enzyme succinoxidase. Therefore, it is suggested that the action of these metals may not be mediated by binding of SH groups in the cells.

Since this work was completed, Passow and Tillmann (1956) have reported that Cd, Zn and Cu have no effect on red cell K^+ at room temperature. The former two we found lead to a small K^+ efflux at 37° C. and it may well be that at room temperature they are ineffective, but it is considered very unlikely that this is the case with Cu, which we found to be as effective as Au at 37° C.

Since K^+ release from the red cells was unassociated with actual cell lysis except in the case of Fe^{+++}, it was prehaemolytic.

The effectiveness of a given concentration of a metal solution on red blood cells was determined, in our experiments, by the absolute amount of metal available to each red cell, rather than by the concentration of metal in the solution. Other workers (Joyce *et al.*, 1954) have expressed the amount of metal added to their cell suspensions as overall concentrations, without relating it to the volume of cells used. Joyce *et al.* (1954) used Pb but hesitated to use a "dosage" type of expression, since the extent of binding of Pb to the red cell was unknown. Our results show that this hesitation was unfortunate since the degree of activity depends not only on the concentration of metal, but also on the number of cells available to receive it. Papers 2 and 3 of the present series will indicate that studies of the extent of binding of Hg and Pb to the human red cell further support the hypothesis put forward here.

Considerable differences between the effects of Hg and Pb were demonstrated, both in regard to the volume changes and to the ionic fluxes. Na^+ influx was much greater under the action of Hg than Pb, but the reverse held for K^+ efflux so that with Pb the nett loss of intracellular electrolytes was greater than with Hg. Cell volume was significantly decreased with Pb, but the decrease was not strictly parallel to the loss of intracellular cations. The loss of cellular water could be secondary to the balance of outward ionic shift in both cases, and the greater loss with Pb could reflect the much more pronounced outward ionic flux induced by this metal. However, after exposure to Hg and Pb the cells were hypotonic in regard to Na^+ and K^+, and more so after Pb than Hg.

The differences between Pb and Hg will be fully discussed when further data are presented (papers 2 and 3 of this series), but it is interesting to compare the above results with Solomon's suggested mechanism of cationic control by the red cell (Solomon, 1952). He suggests that K^+ and Na^+ are each main-

tained constant within the red cell by virtue of independent energy-dependent mechanisms which control a steady state relationship, a state which could be variously disturbed according to whether an inhibitor interfered with one or other mechanisms. It would be in accord with Solomon's theory to suggest that the different effects of Pb and Hg on the K^+ and Na^+ balance of red cells reported here are due to differences in their interference with two distinct ion-transport mechanisms.

SUMMARY.

Pb^{++}, Hg^{++}, Au^{+++}, Cu^{++}, Cd^{++} and Zn^{++} in concentrations of not more than 1 μmoles/ml. of washed red cells suspended in isotonic solution caused a loss of K^+ from these cells without haemolysis.

Mn^{++}, Co^{++}, Ni^{++}, Fe^{+++}, $NaAs_3O_2$ and $NaHAsO_3$ in concentrations of up to 2 μmoles/ml. of red cells did not cause a loss of K^+.

The order of activity of metals tested for activity in releasing K^+ from the red cell was: $Pb^{++} > Hg^{++} > Au^{+++} > Cu^{++} > Cd^{++} > Zn^{++}$.

Certain thiol inhibitors – sodium iodoacetate, sodium tetrathionate, sodium arsenite, and sodium arsenate – were assayed for their effect on permeability of the red cell for K^+, but were without effect.

Pb^{++} and Hg^{++} produced different effects on the red cell, especially in regard to water and electrolyte changes.

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REFERENCES.

Aub, J. C., Fairhall, L. T., Minot, A. S., and Reznikoff, P. (1925): Medicine, 4, p. 1.
Blackburn, C. R. B. (1949): J. biol. Chem., 178, p. 855.
Heilmeyer, L. (1943): Spectrophotometry in Medicine. Translated by A. Jordan. Hilger, London.
Henriques, V., and Ørskov, S. L. (1936): Skand. Arch. Physiol., 74, p. 78.
Jandl, J. H., and Simmons, R. L. (1957): Brit. J. Haemat., 3, p. 19.
Joyce, C. R. B., Moore, Henrietta, and Weatherall, M. (1954): Brit. J. Pharmacol., 9, p. 463.
Ørskov, S. L. (1935): Biochem. Z., 279, p. 250.
Passow, H., and Tillmann, K. (1956): Pfluger's Archiv. Physiol., 262, p. 23.
Solomon, A. K. (1952): J. gen. Physiol., 36, p. 57.
Sutherland, D. A., and Eisentraut, A. M. (1956): Blood, 11, p. 1024.

ALTERATION OF METABOLIC RESPONSE TO ADRENALINE IN MAN BY PRETREATMENT WITH TRIIODOTHYRONINE AND HYDROCORTISONE[1]

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The augmenting effect of thyroid feeding on the various effects of adrenaline and nor-adrenaline in dogs has recently been described (Brewster *et al.*, 1956). Several investigators have previously reported the sensitising influence of thyroid hormones on the effects of adrenaline on the heart (Raab, 1944).

In man much less evidence is available. Horstmann (1954) has described the increased effect of adrenaline on metabolic rate in patients suffering from thyrotoxicosis. In the present study the early effect of triiodothyronine on the metabolic response to adrenaline has been investigated in man. In addition, investigation of the effect of hydrocortisone on the metabolic response to adrenaline has also been carried out in view of the common association of these two hormones in human stress responses (Franksson *et al.*, 1954).

As indicators of the metabolic effect of adrenaline, changes in blood glucose, blood lactate (Cori, 1931; di Salvo *et al.*, 1956) metabolic rate, total nitrogen excretion and nonprotein respiratory quotient (Griffith, 1951) have been used. Observations were also made on blood pressure, pulse rate and various other urinary constituents.

METHODS.

Six healthy volunteer male medical students were studied at weekly intervals on three different occasions. They were in the post-absorptive state having maintained a similar food and fluid intake the previous evening.

The treatments were given in intravenous infusions over a total of 5 hours. For the first 3 hours each student received an infusion of normal saline at the rate of 60 ml./hour.

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This infusion alone served as control; on another occasion hydrocortisone[6] (100 mg. added to 1 litre) was added; while on the third occasion triiodothyronine[7] (0·5 mg) was given as a single intravenous injection at the beginning, the saline being maintained at the same rate as on other occasions. For the fourth hour in addition adrenaline (B.P.) in concentration 5 mg./litre of normal saline was run at the rate of 5 μg./min. from a separate flask. At the end of the fourth hour the adrenaline infusion was stopped and the original infusion was continued for a further hour.

The following observations were made:

(1) Metabolic rate and R.Q. were measured at hourly intervals for 5 hours using the Hartmann-Braun Basal Metabolic Rate Indicator. In each case observations were made at one minute intervals for ten minutes and the mean values taken. Measurements were made during each of 3 hours before the administration of adrenaline, once during the hour of adrenaline administration and within 1 hour after stopping the adrenaline. Subsequent determination of urinary nitrogen output during these periods permitted calculation of the N.P.R.Q.

(2) Blood glucose and lactate at hourly intervals before adrenaline, during adrenaline infusion at 20 minute intervals for 1 hour, and then 40 minutes after stopping adrenaline. Techniques used were those of Nelson *et al.* (1944) for blood glucose and a modification of Barker and Summerson (1947) for blood lactate.

(3) Urinary constituents at hourly intervals for 5 hours—covering the period of 3 hours before, 1 hour during, and 1 hour after adrenaline infusion. These included total nitrogen (micro-Kjeldahl with Nesslerisation) inorganic phosphate, (Taussky and Shorr, 1953) sodium and potassium (EEL flame photometer) and creatinine (Bonsnes and Taussky, 1945).

Before and during the adrenaline infusion observations of pulse rate, blood pressure and the electro-cardiogram were made.

RESULTS.

Effects on blood glucose. The detailed figures are given in Table I. Prior to adrenaline a slight rise was noted with hydrocortisone but no change with triiodothyronine. Adrenaline was associated with a brisk rise in blood glucose. This rise was greater from 40 minutes onwards following triiodothyronine ($P < 0{\cdot}11$) and after discontinuing the adrenaline following hydrocortisone ($P < 0{\cdot}11$). (Fig. 1.)

Effects on blood lactate. The detailed figures are given in Table 2. Prior to adrenaline no significant changes were noted. Adrenaline infusion alone was associated with a rise in blood lactate, which was not altered by pretreatment with hydrocortisone. However pretreatment with triiodothyronine abolished this rise in blood lactate following adrenaline ($P < 0{\cdot}05$). After discontinuing the adrenaline the blood lactate was significantly lower following both hydrocortisone and triiodothyronine when compared with adrenaline alone (Fig 1).

Effects on metabolic rate, N.P.R.Q. and total nitrogen excretion. In each subject adrenaline alone produced a striking increase (approximately 20 p.c.) in metabolic rate without increase in nitrogen excretion or significant change in

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[6] Gift of Upjohn Co., Kalamazoo, Michigan.
[7] Gift of Glaxo Laboratories Ltd., Greenford, Middlesex.

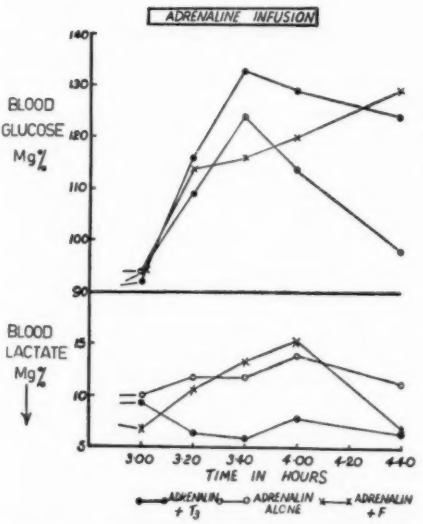

Fig. 1. Changes in blood glucose and blood lactate with adrenaline alone; and following triiodothyronine (T_3), and hydrocortisone (F) pretreatment.

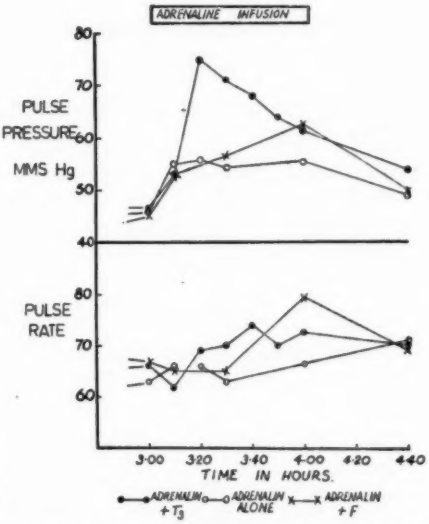

Fig. 2. Changes in pulse pressure and pulse rate with adrenaline alone; and following triiodothyronine (T_3), and hydrocortisone (F) pretreatment.

N.P.R.Q. However, no consistent change was noted following either triiodothyronine or hydrocortisone pretreatment.

Effects on blood pressure and pulse rate. As expected, an increase in pulse pressure due mainly to fall in diastolic blood pressure was noted with the adrenaline infusion alone. This increase was significantly greater following triiodothyronine pretreatment ($P < 0{\cdot}06$). No sustained increase in pulse rate was noted with the adrenaline infusion alone; however such an increase was noted following triiodothyronine pretreatment ($P < 0{\cdot}10$) (Fig. 2). Complete data are available in only 3 instances for hydrocortisone. Increases in pulse pressure had occurred at the end of the infusion in all three subjects while increase in pulse rate occurred in 2 out of 3 subjects when compared with adrenaline alone.

Effects on urine flow, sodium, potassium, inorganic phosphate and creatinine excretion. A fall in urine flow, sodium and potassium excretion was noted during the period of infusion of adrenaline in 3 out of the 4 subjects on whom observations were made. Following hydrocortisone and prior to adrenaline infusion increase in potassium excretion had already occurred in 3 out of the 4 subjects while during the infusion the level of sodium excretion was less and potassium excretion was greater. Increase in inorganic phosphate excretion was noted in 3 out of 4 instances during the period of adrenaline infusion alone. This increase

TABLE 1.

Effect of triiodothyronine (T_3), hydrocortisone (F), saline (P) and adrenaline on blood glucose.

Subject	Treatment	Time in hours and minutes.							
		0	1	2	3	3·20	3·40	4	4·40
M.S.	T_3	82	92	78	93	112	118	133	122
	P	110	95	100	100	120	115	117	95
	F	80	82	80	78	112	108	142	157
A.X.	T_3	108	117	112	110	130	137	137	120
	P	97	87	87	87	115	132	127	115
	F	72	95	92	95	127	127	122	122
B.U.	T_3	95	92	93	82	97	118	118	104
	P	110	104	102	104	102	108	98	82
	F	92	95	97	100	100*	97*	110*	—
B.W.	T_3	110	—	97	87	118	147	122	155
	P	109	98	79	97	92	122	103	93
	F	87	95	110	105	120*	110*	117*	—
W.T.	T_3	95	92	95	87	95	117	95	—
	P	92	97	92	82	132	157	157	125
	F	—	—	—	—	—	—	—	—
L.Z.	T_3+F	72	102	94	92	115	—	155	127
	P	95	110	105	—	117	142	125	105
	F	93	83	88	93	123	123	118	113

* No adrenaline given.

TABLE 2.

Effect of triiodothyronine (T_3), hydrocortisone (F), saline (P) and adrenaline on blood lactate.

Subject	Treatment	Time in hours and minutes							
		0	1	2	3	3·20	3·40	4	4·40
M.S.	T_3	4·6	5·8	3·9	11·5	6·6	8·4	12·7	8·0
	P	8·9	8·8	7·7	9·3	12·8	15·4	14·5	15·8
	F	13·4	9·7	17·7	9·7	4·7	9·4	12·3	7·2
A.X.	T_3	5·0	4·5	3·8	4·3	3·6	3·5	6·8	8·0
	P	—	9·1	6·5	8·5	16·4	11·8	13·6	11·4
	F	9·2	8·1	8·6	5·8	12·7	14·8	17·0	7·5
B.U.	T_3	5·0	5·5	3·4	3·7	2·7	3·7	4·6	2·9
	P	8·2	4·9	4·4	6·2	2·8	5·8	11·3	5·1
	F	7·0	9·6	9·9	11·5	7·7*	9·5*	11·7*	—
B.W.	T_3	8·6	—	8·9	13·4	7·9	6·4	9·3	6·2
	P	6·0	13·5	8·9	15·7	12·7	15·6	18·0	13·6
	F	7·3	7·4	9·6	4·5	6·0*	4·7*	4·8*	—
W.T.	T_3	9·6	12·0	10·1	13·0	11·3	11·7	10·0	6·7
	P	8·3	14·0	9·0	9·9	17·4	17·4	18·7	11·7
	F	—	—	—	—	—	—	—	—
L.Z.	T_3+F	10·1	6·5	7·0	1·05	11·4	—	12·3	8·2
	P	7·2	5·2	8·7	—	8·0	4·4	7·3	8·9
	F	6·2	6·3	4·6	5·7	14·0	11·3	13·0	9·0

* No adrenaline given.

TABLE 3.

Statistical analysis. Blood glucose—Mean values.*

(5 subjects)

Treatment	Time in hours and minutes				
	3·00	3·20	3·40	4·00	4·40
T_3	92	116	133	129	124
P	94	109	124	114	98
F	94	114	116	120	129
T_3 P**	ns	ns	<0·11	<0·10	=0·11
F	ns	ns	ns	ns	<0·10

* Arrived at by calculating missing values for
(1) Subjects B.U. & B.W. who did not receive the adrenaline following hydrocortisone.
(2) Subject L.Z. who received T_3+F and not T_3 alone.
Subject T, whose values were incomplete, was omitted.
** Using "t" test.
ns = not significant.

TABLE 4.

Statistical analysis. Blood lactate—Mean values.*

(6 subjects)

Treatment	Time in hours and minutes				
	3·00	3·20	3·40	4·00	4·40
T_3	9·2	6·4	5·8	7·8	6·4
P	10·0	11·7	11·7	13·9	11·1
F	6·6	10·6	13·2	15·2	6·9
T_3 P**	ns	<0·2	<0·06	<0·05	<0·05
F	ns	ns	ns	ns	<0·05

* Arrived at by calculating missing values for
(1) Subjects B.U. & B.W. & W.T. who did not receive the adrenaline infusion following hydrocortisone.
(2) L.Z. who received T_3+F instead of T_3 alone.
** Using "t" test.
ns = not significant.

was greater following triiodothyronine and persisted after cessation of the adrenaline. Inorganic phosphate excretion was less than with adrenaline alone following hydrocortisone pretreatment. No changes in creatinine excretion were observed.

DISCUSSION.

The pattern of metabolic changes observed following adrenaline alone was in accordance with observations already reported (Griffith, 1951; di Salvo *et al.*, 1956). The brisk rises in blood glucose and blood lactate result from glycogen

breakdown in liver and muscle respectively (Cori, 1931). According to Griffith (1951) the increase in metabolic rate is mainly dependent on changes in the liver-glycogen breakdown and resynthesis from lactate rather than in peripheral tissues. The absence of consistent changes in total nitrogen excretion or fall in N.P.R.Q. in the present investigation suggests carbohydrate sources and would be expected on this basis. The changes associated with adrenaline alone will be discussed further elsewhere.

Following pretreatment with triiodothyronine the lactate increase was abolished, the rise in blood glucose was more sustained while the increase in metabolic rate was unaffected and there was no consistent change in R.Q. These changes may have resulted from a more rapid clearance of lactate by the liver, with increased glycogen synthesis and breakdown producing an elevated blood glucose. There is no evidence of inhibition of peripheral glucose utilisation by thyroid hormones, in fact there is evidence favouring an opposite effect (Mirsky and Broh-Lahn (1936). However, Brewster *et al.* (1956) noted an increase in both the metabolic rate and the lactate response in their thyrotoxic dogs. An explanation for this discrepancy is not apparent though the conditions of the two experiments are hardly comparable.

Following pretreatment with hydrocortisone the lactate increase was unaltered, the rise in blood glucose was more sustained while the increase in metabolic rate was unaffected. Increased deposition of glycogen in the fasting liver is a well-known effect of cortisone (Long *et al.*, 1940). Recent evidence indicates the liver rather than peripheral tissues as the main site of action of hydrocortisone on carbohydrate metabolism (Thorn *et al.*, 1957). An increase in glycogen turnover would therefore seem the likely basis of this increase in blood glucose. The absence of changes in total nitrogen excretion indicates that gluconeogenesis is not a major factor. An increase in glycogen turnover may also be responsible for the rapid fall in lactate following cessation of adrenaline. The similar changes in lactate and glucose after cessation of adrenaline following triiodothyronine suggest a similar explanation.

A fall in sodium and potassium excretion with a tendency to increase in urine flow with adrenaline infusion has been previously reported (Nickel *et al.*, 1954). Similar changes in sodium and potassium excretion were noted in this investigation. The increases in potassium excretion following hydrocortisone and in inorganic phosphate excretion following triiodothyronine have been previously reported (Hetzel *et al.*, 1957) and still occur during the infusion of adrenaline.

The observed increases in pulse pressure and pulse rate following triiodothyronine pretreatment confirm in man the well-known augmenting effects of thyroid hormones on the action of adrenaline on the heart (Raab, 1944; Brewster *et al.*, 1956). Increase in pulse pressure or pulse rate has not been observed with triiodothyronine alone over this period. The changes following hydrocortisone

recall the potentiating effect of ACTH and cortisone on the pressor response of nor-adrenaline (Kurland and Freedberg, 1951) and the potentiation of the pressor effects of nor-adrenaline and adrenaline in man by desoxycorticosterone acetate (Raab *et al.*, 1950).

These results indicate an overall potentiation of adrenaline effects in man by triiodothyronine and hydrocortisone. The importance of adrenaline in man's body responses, particularly those associated with emotion, has been demonstrated by Cannon (1929). Recent studies (Hetzel *et al.*, 1956a, b) disclosed a metabolic reaction pattern associated with emotional stress in man. This pattern consisted of increase in oxygen consumption, fall in R.Q., no change in blood glucose, with increase in urine flow, total nitrogen, sodium and potassium excretion. It is apparent that this pattern has not been reproduced by adrenaline either alone or in combination with hydrocortisone or triiodothyronine.

SUMMARY.

The metabolic effects of adrenaline have been compared following pretreatment with triiodothyronine (0·5 mg) and hydrocortisone (25 mg) given intravenously 3 hours beforehand.

Following pretreatment with triiodothyronine the blood lactate increase was abolished, the rise in blood glucose was more sustained while the increase in metabolic rate was unaffected and there was no consistent change in R.Q.

Following pretreatment with hydrocortisone the lactate increase was unaltered, the rise in blood glucose was more sustained while the increase in metabolic rate was unaffected and there was no consistent change in R.Q.

Potentiation of the haemodynamic effects of adrenaline was also noted following both triiodothyronine and hydrocortisone.

The significance of these changes is briefly discussed.

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REFERENCES.

Barker, S. B., and Summerson, W. H. (1947): From Hawk, P. B., Oser, B. C., and Summerson, W. H., Prac. Physiol. Chemistry, 12th Edition, Philadelphia, Blakiston & Co., 1947.

Bonsnes, R., and Taussky, H. (1945): J. biol. Chem., 158, p. 581.

Brewster, W. R., Isaacs, J. P., Osgood, P. F., and King, T. L. (1956): Circulation 13, p. 1.

Cannon, W. B. (1929): "Bodily Changes in Pain, Hunger, Fear and Rage," New York, Appleton. Second edition.

Cori, C. F. (1931): Physiol. Rev., 11, p. 143.

di Salvo, R. J., Bloom, W. L., Brust, A. A., Ferguson, R. W., and Ferris, E. G. (1956): J. clin. Invest., 35, p. 568.

Franksson, C., Gemzell, C. A., and von Euler, U. S. (1954): J. clin. Endocrin. Met., 14, p. 608.

Griffith, F. R. (1951): Physiol. Rev., 31, p. 151.

Hetzel, B. S., Grace, W. J., and Wolff, H. G. (1956a): J. psychosom. Res., 1, p. 186.
Hetzel, B. S., Schottstaedt, W. W., Grace, W. J., and Wolff, H. G. (1956b); *Ibid.*, 1, p. 177.
Hetzel, B. S., Williams, R., and Lander, H. (1957): Australasian Ann. Med., 6, p. 218.
Horstmann, P. (1954): Acta Endocrinologica, 16, p. 233.
Kurland, G. S., and Freedberg, A. S. (1951): Proc. Soc. exp. Biol. Med., 78, p. 28.
Long, C. N. H., Katzin, B., and Fry, E. G. (1940): Endocrinology, 26, p. 309.
Mirsky, I. A., and Broh-Lahn (1936): Amer. J. Physiol., 117, p. 6.
Nelson, N. (1944): J. biol. Chem., 153, p. 375.
Nickel, J. F., Smythe, C. McC., Papper, E. M., and Bradley, S. E. (1954): J. clin. Invest., 33, p. 1687.
Raab, W. (1944): J. Pharmacol. exp. Ther., 82, p. 330.
Raab, W., Humphreys, R. J., and Lepechkin, E. (1950): J. clin. Invest., 29, p. 1397.
Taussky, H. H., and Shorr, E. (1953): J. biol. Chem., 202, p. 675.
Thorn, G. W., Renold, A. E., and Winegrad, A. I. (1957): Brit. med. J., 2, p. 1009.

EFFECTS OF AMARIN, 4-METHYL-2-AMINO-PYRIDINE ('W-45'), TETRAETHYL-UREA, AND BEMEGRIDE ON MICE GIVEN TOXIC DOSES OF PENTOBARBITONE SODIUM

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Studies concerned primarily with the circulatory properties of various ureas, amidines, and related compounds drew our attention to the central stimulant action of certain of the compounds tested. Convulsant and related actions of amarin, tetraethyl-urea (TEU) and 4-methyl-2-amino-pyridine (W-45) have been mentioned in earlier papers (Fastier and Smirk, 1948 and 1956; Fastier and McDowall, 1958). The analeptic action of TEU has been noted also by Aeschlimann (1936) and that of W-45 by von Haxthausen (1955). The hope that at least one of these compounds might be a useful barbiturate antagonist induced us to perform the following experiments.

EXPERIMENTAL.

Mice were given doses of pentobarbitone sodium which killed about half of each control group (Table 1). Doses of 110, 125 and 140 mg./kg. intraperitoneally killed on the average 35, 50 and 66 p.c. respectively.

As β-ethyl-β-methyl-glutarimide (Bemegride, 'Megimide') has been shown to be a good barbiturate antagonist both experimentally and clinically (Shaw, 1957), this drug was tested in addition to W-45, TEU and amarin. Bemegride clearly increased the survival rate in all 3 experiments in which it was employed (Table 1). Moreover, it shortened sleeping times; treated mice slept only about half as long as the mice of the control groups.

None of the other compounds tried had a comparable effect. In only 1 out of 20 experiments was the survival rate for the treated group much greater than that for the control group. Under most of the conditions tried (Table 1) treatment with W-45, TEU or amarin afforded no protection. In some experiments considerably more treated mice than controls succumbed. It was observed repeatedly with W-45 that the treated mice did not recover so quickly as the controls, despite the fact that they were breathing better and appeared to be considerably 'lighter' for some ten or fifteen minutes after the injection of the analeptic. Presumably central stimulation was followed by after-depression. The

TABLE 1.

Effects of 4-methyl-2-amino-pyridine (W-45), tetraethyl-urea (TEU), bemegride ("Bem.") and amarin ("Amar.") on the survival rates of mice given various doses of pentobarbitone sodium.

Dose[1] of hypnotic	Treatment				Survivors/total in group	
	Drug	Dose[1]	Route[2]	Time[3]	Treated group	Controls
140	Bem.	100	s.c.	10	18/20	9/20
140	W-45	100	s.c.	10	14/20	12/20
140	W-45	100	i.p.	10	2/20	2/20
140	W-45	100	s.c.	15	1/10	1/10
140	W-45	150	s.c.	15	7/20	8/20
140	TEU	100	i.p.	15	4/20	10/20
140	TEU	150	s.c.	15	9/20	4/20
140	TEU	200	s.c.	10	9/20	7/20
140	Amar.	25	i.p.	15	3/10	2/10
125	Bem.	100	i.p.	10	19/20	12/20
125	Bem.	100	i.p.	20	19/20	13/20
125	W-45	100[4]	i.p.	15	6/10	0/10
125	W-45	100[4]	i.p.	5	13/20	14/20
125	W-45	70	i.p.	10	5/20	6/20
110	W-45	70	i.p.	2	12/20	18/20
110	W-45	50	i.p.	15	14/20	13/20
110	TEU	200	i.p.	10	0/10	5/10
110	Amar.	25	i.p.	15	4/5	3/5
110	Amar.	25	i.p.	2	10/10	8/10
110	Amar.	50	i.p.	15	0/5	2/5
110	Amar.	50	i.p.	2	0/5	0/5
80	Amar.	50	i.p.	15	3/5	4/5
80	Amar.	75	i.p.	15	0/5	4/5

[1] in mg./kg. [2] intraperitoneal (i.p.) or subcutaneous (s.c.)
[3] interval in minutes between injection of barbiturate and analeptic.
[4] in two 50 mg./kg. doses 2 hours apart.

convulsant action of amarin was more conspicuous than that of the other compounds under these conditions; it was also much more persistent. For this reason experiments were performed in which amarin was tested against long-acting barbiturates.

When given to rats which had received thirty minutes previously a dose of barbitone sodium (330 mg./kg. intraperitoneally) which proved close to the LD_{80}, amarin in moderate doses (50-75 mg./kg. intraperitoneally) definitely increased the survival rate. In 6 experiments similar to those performed on mice, 31 in all of 77 treated rats survived compared with only 12 of the 77 controls. Twitchings or frank convulsions were produced in nearly all of the amarin-treated rats, sometimes for as long as twelve hours. Amarin did not advance the waking time to a noteworthy extent even in twitching rats. Survivors showed no signs of delayed toxicity when killed several weeks later.

Amarin (50, 75 mg./kg.) did not protect rats which had been given larger doses (500, 750 mg./kg.) of barbitone sodium. Larger doses of amarin (120, 150 mg./kg.) did not increase the survival rate for rats which had been given the usual dose (330 mg./kg.) of barbitone sodium. Amarin (50, 100 mg./kg.) did not increase to a significant extent the survival rate of rats which had been given a dose of phenobarbitone sodium approximating to the LD_{70}. This barbiturate prevented convulsions in the amarin-treated rats.

SUMMARY.

The proportion of mice dying as a result of their being given a dose of pentobarbitone sodium approximating to the LD_{50} was substantially reduced by bemegride given 10 or 20 minutes later in a dose of 100 mg./kg. either subcutaneously or intraperitoneally. The proportion was not reduced in most experiments by amarin (25, 50, 75 mg./kg.), tetraethyl-urea (100, 150, 200 mg./kg.) or 4-methyl-2-amino-pyridine (50, 70, 100, 150 mg./kg.) injected at various times after the pentobarbitone.

The survival rate for rats given a dose of barbitone sodium approximating to the LD_{80} was more than doubled by treatment with moderate doses (50-75 mg./kg.) of amarin. However, under most of the conditions chosen, amarin did not protect rats against barbitone sodium or phenobarbitone sodium to a significant extent.

It is concluded that none of the other three compounds tested can match bemegride as a barbiturate antagonist.

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REFERENCES.

Aeschlimann, J. A. (1936): Festschrift Emil C. Barell, Reinhardt, Basle, p. 246.
Fastier, F. N., and McDowall, M. A. (1958): Austral. J. exp. Biol., 36, p. 365.
Fastier, F. N., and Smirk, F. H. (1948): J. Physiol., 107, p. 318.
Fastier, F. N., and Smirk, F. H. (1956): Proc. Univ. Otago med. Sch., 34, p. 21.
Haxthausen, E. von (1955): Arch. exp. Path. Pharmak., 226, p. 163.
Shaw, F. H. (1957): Med. J. Austral., I, p. 712.

ANALGESIC ACTIVITY OF 4-METHYL-2-AMINO-PYRIDINE AND OF SOME RELATED COMPOUNDS

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Having observed that the 2-, 3-, and 4-amino-pyridines possess both pressor and central stimulant activity, von Haxthausen (1955a) tested a number of related compounds for similar properties. He concluded that the best of these as an analeptic was 4-methyl-2-amino-pyridine ('W-45'). Further experiments (Haxthausen, 1955b) disclosed the surprising fact that W-45 possesses strong analgesic activity – almost 70 p.c. that of morphine as judged from tests involving the application of graded thermal or electrical stimuli to rats, mice and rabbits. The analgesic action of W-45 was antagonized by nalorphine.

For reasons given in an earlier paper (Fastier and McDowall, 1958), we have already investigated certain pharmacological properties of the amino-pyridine isomers. Since this study led to our obtaining a number of related compounds, we have taken the opportunity of seeing whether any of these resemble W-45 in possessing analgesic as well as pressor and central stimulant activity.

METHODS.

Analgesic effects were studied by three techniques: (1) a hot plate method which closely resembled that employed by Woolfe and McDonald (1944), (2) the use of 2-phenyl-1:4-benzoquinone to produce in mice a 'squirming syndrome' which is known to be allayed by typical analgesics but not by such drugs as hypnotics and tranquillizers (Siegmund, Cadmus and Lu, 1957), and (3) a tail clip method similar to that devised by Bianchi (1956) for the assessment of local anaesthetic activity.

As hot plate we used a large copper beaker, 15 cm. high, 10·5 cm. in diameter, and maintained at a constant temperature by a water bath. Mice (females, 25-30 gm.) for use in hot plate experiments were accustomed to respond to the stimulus by placing them one by one in the hot beaker. The time taken for each mouse to respond by jumping out of the beaker or on to the rim was measured. Mice not jumping out within 30 sec. were considered not to respond and were removed from the beaker. After three preliminary trials in the hot beaker at 60° C., all mice jumped out in less than 10 sec. The analgesic was then given subcutaneously in the back. Subsequent tests of the responses of the mice were made at 10 min. intervals for up to 90 min. after injection.

The 2-phenyl-1:4-benzoquinone was injected intraperitoneally in a fixed dose (60 μg. dissolved in 0·25 ml. of isotonic NaCl containing 5 p.c. ethanol). It was given to batches

of 10 female mice weighing 20-25 gm. The number of mice in each batch which squirmed at least once in a 5 min. interval was counted for successive 5 min. intervals from 10 min. after the injection of the benzoquinone up to an hour later. The substance to be tested for analgesic activity was usually given subcutaneously in the 10-15 min. interval after the injection of the benzoquinone.

Local anaesthetic effects were studied by (1) the method of Bülbring and Wajda (1945), in which small weals raised in the depilated backs of guinea-pigs by the intracutaneous injection of the solutions under test are pricked at regular intervals, and (2) the method of Bianchi (1956), in which a small artery clip is placed near the base of a mouse's tail at intervals before and after the subcutaneous injection of the drug into the tail.

Toxicity measurements were made on female mice weighing 25-30 gm. and kept in individual beakers at a temperature of 21° C. Experiments on anaesthetized cats were performed as described in an earlier paper (Fastier and McDowall, 1958).

The pK_a values of the amino-pyridines were determined by potentiometric titrations involving the use of a 'Radiometer' pH meter. The pH of the solution was measured at intervals when 10 ml. of an M/60 solution of the amino-pyridine was gradually neutralised with M/20 hydrochloric acid. The end-point was determined graphically from a plot of pH versus titre and the pK_a value taken as the pH at half the end-point titre, calculated by linear interpolation. Values thus obtained were corrected for dilution to give 'thermodynamic pK_a values'. A correction of 0·09 pH unit was made for the end-point concentration of M/70 (Albert, Goldacre and Phillips, 1948).

All the pyridine derivatives tested were purchased from L. Light and Co. Aqueous solutions for injection were made approximately isotonic by the addition of sodium chloride. They were neutralised with acetic acid. The benzoquinone (synthesized by M.A.M.) was prepared for injection by dissolving 6 mg. in 1 ml. hot ethanol and adding 24 ml. of isotonic sodium chloride solution. The resulting solution was kept hot prior to injection to prevent precipitation of the benzoquinone.

RESULTS.

Toxicity.

The toxic effects of the 3-methyl-, 4-methyl-, 5-methyl-, 6-methyl-, and 4:6-methyl derivatives of 2-amino-pyridine resembled those of the 2-, 3-, and 4-amino-pyridines, tested previously (Fastier and McDowall, 1958). The subcutaneous or intraperitoneal injection of a fatal dose produced tremors, followed by violent convulsions and death.

The LD_{50} values estimated for these compounds from the death rates found in three batches of ten mice given graded doses subcutaneously in the back were:

3-methyl-2-amino-pyridine ('3-Me-2-AP')	36 mg./kg.;
4-methyl-2-amino-pyridine ('W-45')	80 mg./kg.;
5-methyl-2-amino-pyridine ('5-Me-2-AP')	110 mg./kg.;
6-methyl-2-amino-pyridine ('6-Me-2-AP')	52 mg./kg.;
4:6-dimethyl-2-amino-pyridine ('Me_2-2-AP')	25 mg./kg.

Similar fatal convulsions were produced in mice by 2:6-diamino-pyridine (100 mg./kg. subcutaneously), but not by 2-benzylamino-pyridine, 2:6-diacetylamino-pyridine, or 5-chloro-2-amino-pyridine in doses of up to 200 mg./kg.

Analgesic activity.

Hot plate method. The results of two typical experiments are given in Table 1. In the first experiment, two batches of ten mice which had been 'trained' in the three preliminary trials to leap out of the hot beaker within 15 sec. of their being placed in it were given W-45 (25 mg./kg. subcutaneously) and isotonic sodium chloride (0·25 ml.) respectively. It can be seen that, whereas all the mice of the control group continued to respond quickly to the stimulus except towards the end of the experiment, a considerable proportion of the treated mice did not – presumably because of an analgesic action of W-45. In the second of the experiments illustrated in Table 1, the mice of

TABLE 1.

Analgesic effects of W-45 and morphine on mice evaluated by the "hot plate" method.

Treatment			Number of mice in a batch of 10 which did not jump out of the hot beaker within the time stated (either 15 or 30 sec.).									
			Trials			Time after injection (min.)								
Drug	Dose (mg./kg.)	Time (sec.)	I	II	III	10	20	30	40	50	60	70	80	90
Saline control	—	15	10	1	0	0	0	0	0	0	1	1	1	4
		30	4	1	0	0	0	0	0	0	0	1	1	2
W-45	25	15	9	1	0	4	5	4	5	4	2	1	3	5
		30	6	0	0	1	3	3	4	3	0	1	3	4
Morphine HCl	4	15	5	2	0	2	4	7	8	8	7	6	7	4
		30	5	1	0	2	4	6	4	6	7	6	6	3
W-45	25	15	6	2	0	8	8	6	7	5	5	1	5	1
		30	5	0	0	5	5	6	5	5	4	0	2	1

one batch were treated with W-45 (25 mg./kg. subcutaneously) and those of the other batch with morphine hydrochloride (4 mg./kg.). As in other experiments in which both drugs were given, W-45 acted more quickly than morphine and had a less persistent effect. Whereas the maximum effect of W-45 was obtained within 20 min. of the injection of the drug, that of morphine was not obtained until some 30-50 min. had elapsed. W-45 had no detectable effect in a dose of 10 mg./kg. and very little effect in a dose of 15 mg./kg. Morphine hydrochloride had a much more powerful effect than W-45 when given in the same dose by weight. For example, a dose of 20 mg./kg. so affected all the mice of a batch that they remained within the hot beaker for 30 sec. whenever they were placed in it during the period of observation (20-90 min. after the injection of the morphine).

Quinone method. A 60 μg. intraperitoneal dose of 2-phenyl-1:4-benzoquinone produced characteristic squirming movements. With individual mice the rate of squirming was highly variable. However, the proportion of mice which squirmed at least once during a particular 5 min. interval after the injec-

tion of quinone did not vary much from one batch to another. The results obtained with the control groups in the first four experiments are given in Table 2. No observations of the number of mice squirming during the 20-25 min. interval were made in these four experiments because the other mice used in the experiment were given either morphine or W-45 during this period. Subsequently, the compound under test was given sooner after the injection of the quinone because the impression was gained that the best time to test the effects of analgesics was 15-35 min. after the injection of the quinone. During this 20 min. period approximately 75 p.c. of the mice would be expected to squirm at least once every five minutes if they received no drug which antagonized the effect of the quinone (Table 2).

W-45 abolished the squirming entirely when given subcutaneously in a dose of 20 mg./kg. It was almost as effective in a dose of 10 mg./kg., but definitely less effective in a dose of 5 mg./kg. (Table 3). Morphine took much

TABLE 2.

Production of squirming in mice by the intraperitoneal injection of 2-phenyl-1:4-benzoquinone (60 μg.).

Total No. in batch	No. in batch squirming at least once within the stated time interval (in min.) after treatment								
	15-20	20-25	25-30	30-35	35-40	40-45	45-50	50-55	55-60
11	8	—	8	8	6	4	4	6	4
11	8	—	8	8	7	6	4	5	7
10	6	—	8	10	7	4	5	4	2
10	7	—	7	8	6	5	3	4	2
Mean for 10 (females)	6·9	—	7·4	8·1	6·2	4·5	3·8	4·5	4·1
20	16	16	13	11	15	11	12	9	9
20	17	18	17	14	15	16	13	9	8
Mean for 10 (males)	8·3	8·5	7·5	6·3	7·5	6·8	6·3	4·5	4·3

TABLE 3.

Antagonism of the squirming "syndrome" produced by 2-phenyl-1:4-benzoquinone by W-45.

Dose of W-45 mg./kg.	No. of mice in batch of 10 squirming at least once during the specified 5 min. interval after the injection of the quinone. W-45 was given at "Inj.".								
	10-15	15-20	20-25	25-30	30-35	35-40	40-45	45-50	50-55	55-60
Controls (mean)	—	7	7	7	8	6	5	4	5	4
5	—	10	Inj.	5	2	3	6	5	4	5
10	7	9	Inj.	1	0	0	1	1	1	1
20	—	10	Inj.	0	0	0	0	0	0	0

TABLE 4.

Antagonism by the amino-pyridines and related compounds of the squirming produced by 2-phenyl-1:4-benzoquinone.

Compound[1]	pK[2]a	LD^{3}_{50}	Dose[4]	Number in batch of 10 mice squirming at least once in the period:			
				20-25	25-30	30-35	total
(Mean for controls)	—	—	—	8	7	7	22
2-AP	6·86	(70)	4	6	3	6	15
			14	1	1	3	5
			17·5	1	0	0	1
			35	0	0	0	0
3-AP	6·07	(35)	8	4	4	3	11
			11·5	1	1	2	4
			14	0	0	0	0
4-AP	9·31	(5)	1	3	4	5	12
			1·5	0	0	0	0
			2·5	0	0	0	0
3-Me-2-AP	7·24	36	10	0	4	5	13
			14	2	3	3	8
4-Me-2-AP	7·48	80	4	6	3	6	15
			5·5	4	4	4	12
			6·5	2	1	3	6
5-Me-2-AP	7·22	110	30	2	3	2	8
			35	2	4	3	9
			45	3	2	2	7
6-Me-2-AP	7·41	52	15·5	5	5	5	15
			20	4	4	2	10
			27	0	0	0	0
Me_2-2-AP	7·84	25	7·5	3	2	2	7
			10	0	0	1	1

[1] See text for explanation of code names.
[2] Determined as indicated in text.
[3] By subcutaneous route. Values in brackets were those obtained by Haxthausen (1955a).
[4] In mg./kg.

longer than W-45 to exert its full effect. It acted more powerfully, however. A 1 mg./kg. subcutaneous dose of the hydrochloride abolished the squirming syndrome in 7 out of 10 mice within 15 min. and in all 10 mice within 30 min. A 0·5 mg./kg. dose of morphine hydrochloride was less effective than a 10 mg./kg. dose of W-45 in antagonizing the action of the quinone.

In subsequent experiments with W-45 and other pyridine derivatives, the incidence of squirming was noted during the 20-35 min. period only (Table 4). All the compounds tested showed some analgesic activity. Each was given in several doses, the aim being to find a dose (the 'ED_{70}') which would reduce by some 70 p.c. the number of mice which squirmed at least once during the 20-35

min. period after the injection of the quinone. Although W-45 proved to be a less powerful analgesic than 4-amino-pyridine, it has a better therapeutic index (as judged by dividing the LD_{50} by the ED_{70}) than the latter or any other of the compounds tested. To see whether an ability to antagonize the action of the quinone is even more widespread, 2 experiments were performed in which S-methyl *iso*thiouronium sulphate was given in a dose of 200 mg./kg. (approximately one-quarter of the LD_{50} for mice by the subcutaneous route). This dose of the *iso*thiourea was found to be close to the ED_{70} (as just defined).

Tail clip method. Usually untreated mice reacted within 10 sec. to the placing of a light artery clip near the base of the tail by turning their head round to investigate; most of them then tried to remove the clip by biting at it. Some 20-30 p.c. did not react within 30 sec. These mice were rejected. When 20 suitable mice were given a 10 mg./kg. dose of W-45 subcutaneously in the back, 14 of them did not react within 30 sec. to the placing of the clip on their tail 10 min. after the administration of the drug. In an experiment in which the dose of W-45 was reduced to 8·5 mg./kg., 10 of a batch of 20 mice did not react. In another in which it was reduced to 7·5 mg./kg., only 2 of a batch of 20 mice did not react within 30 sec.

Local anaesthetic activity.

When the tail clip method was used for assessing local anaesthetic activity, W-45 and other drugs were injected subcutaneously into the tail beyond the clip, not into the back. The number of mice which bit at the clip within 30 sec. was reduced to 7 out of 20 when 0·1 ml. of a 2 p.c. solution of W-45 was tested, and to 5 out of 14 when 0·1 ml. of a 1 p.c. solution of W-45 was injected. A similar reduction was obtained when procaine was given in the same dosage. However, as W-45 is rapidly absorbed from various sites, and as the amounts injected into the tail were as large as those found to produce a substantial degree of analgesia when injected subcutaneously in the back, it was not clearly established that failure to bite at the clip within the specified period was due to a local anaesthetic as distinct from an analgesic action of W-45.

Guinea-pigs responded less frequently to the pricking of weals raised in their backs by the intracutaneous injection of a solution of W-45 than they did to the pricking of weals raised by the injection of saline. In a typical experiment, pricking caused contraction of the surrounding skin in 126 out of 144 trials made on the control weals during a 45 min. period, but in only 35 out of 144 trials made on weals raised by injecting 0·25 ml. amounts of a 1 p.c. solution of W-45. Procaine given in the same amount was completely effective in inhibiting the response to pricking for at least 45 min. after its injection. The effect of W-45 in these experiments was undoubtedly a local one, since each guinea-pig received at one and the same time intracutaneous injections of both W-45 and saline solutions; were W-45 inhibiting the response to pricking by virtue of a central action, then the same effect would have been seen for both types of weal.

Other properties.

The effects of W-45 on anaesthetized cats, perfused rat hind-quarters, and rat phrenic nerve-diaphragm preparations were more powerful and persistent than, but otherwise similar to, those of 2-amino-pyridine, tested previously (Fastier and McDowall, 1958). The other methyl derivatives of 2-amino-pyridine were less active than W-45 in raising blood pressure and stimulating respiration. Whereas W-45 in a dose of 1 mg./kg. intravenously raised the arterial blood pressure of anaesthetized cats by some 60-70 mm. of mercury on the average, other methyl derivatives of 2-amino-pyridine raised blood pressure by only some 10-30 mm. No effects of note were produced by 5-chloro-2-amino-pyridine.

DISCUSSION.

Mice which have received adequate doses of morphine or of W-45 react much less readily than do ordinary mice to the presumably unpleasant situation created when they are placed in a hot beaker, have their tail squeezed by an artery clip, or receive an injection of 2-phenyl-1:4-benzoquinone. It is likely that the effects of these drugs on mice are brought about by analgesic actions. We cannot be certain that W-45 possesses analgesic activity until it has been shown that W-45 is able, like morphine, to alleviate pain in man. However, while the evidence that W-45 possesses analgesic activity is indirect, it is strong. Like von Haxthausen (1955b), we have obtained concordant results with dissimilar techniques, each of which has been found empirically to be of value for assessing analgesic activity.

Our estimate of the analgesic potency of W-45 is considerably less than von Haxthausen's. A reason for this discrepancy may be found in our observation that W-45 has a more rapid and a less persistent action than morphine. A comparison of the analgesic effects of the drugs made within 10-15 min. after their injection would suggest that W-45 acts almost as strongly as morphine. If, however, the maximum effect of W-45 is compared with the maximum effect of morphine (obtained later in the experiment), then it would appear that W-45 is only about one-tenth as active as morphine on a dosage basis. Nevertheless, even this degree of analgesic activity is remarkable in a compound of fairly simple structure. W-45 and morphine have hardly any structural features in common. Support for the idea that they act at the same site is provided by the finding (Haxthausen, 1955b) that nalorphine antagonizes the analgesic action of W-45, but a great deal more evidence would be required to make this idea attractive. It is possible that W-45 and morphine act at different sites though producing similar effects.

Since chemical relatives of W-45 can be obtained much more easily than chemical relatives of morphine, we have tried to discover what structural or physical attributes of the W-45 molecule are of importance for the possession of analgesic activity. Analgesic activity, as indicated by ability to suppress the

squirming caused by the injection of 2-phenyl-1:4-benzoquinone in mice, is displayed not only by several methylated derivatives of 2-amino-pyridine, but also by 2-amino-pyridine itself and by 3- and 4-amino-pyridine. Clearly analgesic activity is not highly specific from a structural standpoint. As the amino-pyridines tested differ considerably in basicity, their pK_a values were measured as well as their analgesic activity. It may be seen from Table 4 that the most active compound (4-AP) was also the most highly basic, but no close correlation between analgesic activity and basicity was found. Unfortunately, the least basic of the compounds available (5-chloro-2-amino-pyridine, with a pK_a of 4·83) was too insoluble to be tested satisfactorily for analgesic activity. This compound showed no resemblance to W-45 in experiments on anaesthetized cats.

We think it significant that the ratio of ED_{70} (as already defined) to the LD_{50} does not vary much from one compound to another. This suggests that the lethal action of typical amino-pyridine derivatives is brought about by the same fundamental mechanism as their analgesic action. There is an alternative explanation, however. Some compounds may be both more analgesic and more toxic than others merely because they are able to reach their sites of action in the central nervous system in higher concentration for a given subcutaneous dose.

SUMMARY.

It has been confirmed that 4-methyl-2-amino-pyridine ('W-45'), a compound much less complex in structure than morphine, possesses considerable analgesic activity. Results obtained by 3 dissimilar techniques indicate that W-45 has about one-tenth of the analgesic activity of morphine. W-45 has slight local anaesthetic activity. Its circulatory properties resemble those of 2-amino-pyridine and its isomers.

Several other amino-pyridine derivatives possess comparable analgesic activity. On a weight basis, 4-amino-pyridine acts more powerfully than W-45. However, none of the other compounds tested acted as powerfully as W-45 when given in the same fraction (e.g., one-fifth) of the LD_{50}.

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REFERENCES.

Albert, A., Goldacre, R. J., and Phillips, J. (1948): J. chem. Soc., p. 2240.
Bianchi, C. (1956): Brit. J. Pharmacol., 11, p. 104.
Bülbring, E., and Wajda, I. (1945): J. Pharmacol., 85, p. 78.
Fastier, F. N., and McDowall, M. A. (1958): Austral. J. exp. Biol., 36, p. 365.
Haxthausen, E. von. (1955a): Arch. exp. Path. Pharmak., 226, p. 163.
Haxthausen, E. von (1955b): *Ibid.*, 227, p. 234.
Siegmund, E., Cadmus, R., and Lu, G. (1957): Proc. Soc. exp. Biol. Med., 95, p. 729.
Woolfe, G., and McDonald, A. D. (1944): J. Pharmacol., 80, p. 300.

THE THROMBOPLASTIC COMPONENT OF INTACT BLOOD PLATELETS IS PRESENT IN MASKED FORM

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One of us (P.F.) recently reported that the rate of thrombin formation in diluted horse blood was lower than in normal human blood, although the yield of thrombin was the same in both cases. The essential clotting factors showed similar activities and the number of platelets was of the same order in the blood of the two species. As platelets are thought to provide phospholipids (P-lipids) for the formation of blood thromboplastin, and, since the addition to horse blood of a P-lipid fraction isolated from brain increased the rate of thrombin formation considerably, it was suggested that the platelets of horse blood are more stable than those of human blood (Fantl and Marr, 1958). It seemed desirable, therefore, to investigate the stability of human platelets during the clotting process.

MATERIALS AND METHODS.

Venous blood was used and the first few ml. obtained were discarded and the rest drawn into silicone-coated syringes. Oxalated blood was obtained by mixing one volume of 0·1M sodium oxalate and nine volumes of blood.

Russell's viper venoms (R.V.V.) were commercial preparations (Burroughs Wellcome & Co.) which had been stored at 6° C. for 10-12 years. They had approximately 10 p.c. of the activity of fresh R.V.V. preparations. 0·1 mg. of venom was dissolved in 1 ml. 0·145M sodium chloride prior to use.

A P-lipid fraction was isolated from acetone-dehydrated brain essentially according to Eagle (1935).

General Electric "Dri-film 9987" was used for siliconing glassware.

Platelets were counted by a technique similar to that of Feissly and Lüdin (1949).

Organic phosphorus was determined by the technique of Allen (1940). The approximate weight of P-lipid was calculated as $P \times 25$.

The rate of thrombin formation was determined according to Fantl (1954).

RESULTS.

A comparison between the rate of thrombin formation in diluted normal human blood and horse blood is given in Fig. 1.

[1] This work was carried out with the support of a grant from the National Health and Medical Research Council, Canberra.

Fig. 1 shows that the rate of thrombin formation in horse blood was considerably lower than in normal human blood and was comparable to that in platelet-deficient human blood. The addition of P-lipid to diluted blood produced a higher rate of thrombin formation.

In order to introduce only minimal changes in the blood composition experiments were carried out with whole blood. In Table 1 are recorded clotting times of normal human blood, of blood from patients with a deficiency of platelets and of normal blood to which P-lipids were added, in glass and silicone-coated tubes.

The results in Table 1 show that the clotting times of normal and thrombocytopenic blood are of the same order. The addition of P-lipid reduced the clotting time in all instances. The amount of P-lipid added to B was approximately one-quarter of that which is normally present in the platelets of 1·5 ml. blood.

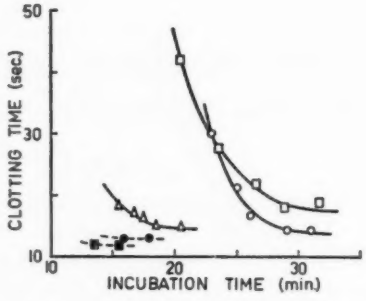

Fig. 1. Rate of thrombin formation in human and horse blood.
1. Normal human blood containing 19 × 10^4 platelets per mm^3. △——
2a. Blood of a patient containing 2 × 10^4 platelets per mm^3. □——
2b. Same as 2a with addition of 30 μg. P-lipid. ■- - - -
3a. Blood from 5-year-old stallion containing 18 × 10^4 platelets per mm^3. ○——
3b. Same as 3a with addition of 30 μg. P-lipid. ●- - - -

In each case 0·04 ml. freshly drawn oxalated blood was used, to which was added 0·86 ml. 0·145M NaCl, 0·3 ml. 0·18M pyrocatechol in veronal buffer, pH 7·3, and 0·6 ml. 0·025M $CaCl_2$. Thrombin was determined with human Ba-plasma, according to Fantl (1954).

In further experiments clotting times of whole blood were determined in the presence of R.V.V. because it is known that certain lipids shorten the R.V.V. clotting time (Trevan and Macfarlane, 1938).

Since it is probable that the active compounds in the platelets necessary for thromboplastin formation are of P-lipid nature, experiments with intact platelets should indicate the availability of the platelet factor for activation of R.V.V.

The results in Table 2 indicate that despite the marked difference between the clotting times of blood diluted with sodium chloride solution in glass and silicone-coated tubes the clotting times in the presence of R.V.V. are practically the same. Portions of the blood specimens referred to in Table 2 were oxalated and plasmas with high and low platelet counts prepared by low and high speed centrifugation. These plasmas were clotted under the experimental conditions given in Table 3.

It will be noted (Tables 2 and 3) that there is a difference between clotting times of whole blood, platelet-rich plasma and platelet-poor plasma in the

TABLE 1.

*Clotting times of human blood**.

	No. of platelets per mm.^{3} blood $\times 10^{-4}$	Clotting time (sec.)	
		In glass	In silicone
Normal	15-30	720-1300	2400-5400
Patient P_1	2·0	840	4200
Patient P_2	0·8	780	2400
Normal A	20	780	3100
Normal A + P-lipid	20	175	240
Normal A + NaCl	20	630	1800
Normal B	25	900	3950
Normal B + P-lipid	25	150	500
Normal B + NaCl	25	380	2000

*1·5 ml. venous blood was used.

The addition consisted of either 0·15 ml. 0·145M NaCl or 0·15 ml. P-lipid suspension in 0·145M NaCl.

To blood A was added 1·5 mg. P-lipid and to blood B 0·027 mg. Temp. 37° C.

TABLE 2.

*Blood clotting times in the presence of Russell's viper venom** (*R.V.V.*)

		No. of platelets per mm.^{3} blood $\times 10^{-4}$	Clotting time (sec.)	
			In glass	In silicone
1	1·6 ml. normal human blood + 0·4 ml. 0·145M NaCl	20	270	960
	1·6 ml. normal human blood + 0·4 ml. R.V.V.	20	85	75
2	1·6 ml. normal human blood + 0·4 ml. 0·145M NaCl	26	320	1300
	1·6 ml. normal human blood + 0·4 ml. R.V.V.	26	60	58

*The determinations were carried out at 37° C.

TABLE 3.

*Influence of P-lipid and R.V.V. on plasma clotting in glass before and after freezing.**

		No. of platelets per mm.^{3} plasma $\times 10^{-4}$	Clotting time (sec.)
1	Plasma + NaCl	35	35
	Plasma + P-lipid	35	10
	Plasma + NaCl	2·5	39
	Plasma + P-lipid	2·5	10
2	Plasma + NaCl	46	36
	Plasma + P-lipid	46	7
	Plasma + NaCl	1·2	42
	Plasma + P-lipid	1·2	7
1a	Frozen and thawed platelet-rich plasma 1 + NaCl	—	9·0
	Frozen and thawed platelet-rich plasma 1 + P-lipid	—	7·5

* Platelet-rich plasma was obtained by centrifugation at 190*g* at 4° C. for 15 min. Platelet-poor plasma was obtained by centrifugation at 3000*g* at 4° C for 30 min. Portion of the platelet-rich plasma 1 was frozen, kept at −15° C for 5 min., then thawed. 0·1 ml. oxalated plasma, 0·1 ml. R.V.V. and either 0·1 ml. 0·145M NaCl or 0·1 ml. P-lipid suspension in 0·145M NaCl containing 30 μg. P-lipid were incubated for 1 min. at 37° C; 0·1 ml. 0·025M $CaCl_2$ was then added and the clotting time measured.

presence of R.V.V. The addition of P-lipid to platelet-rich and platelet-poor plasma reduced the R.V.V. clotting time very considerably. On the other hand, the R.V.V. clotting time of frozen and thawed platelet-rich plasma was very short and could be further shortened only to a very small degree by the addition of P-lipid. It should be mentioned that freezing and thawing had no influence on the clotting time of platelet-free plasma. 30 μg P-lipid were added, which is approximately twice the amount present in the platelets of 0·1 ml. plasma containing 35×10^4 platelets per mm.^{3}

DISCUSSION.

Sufficient clinical and experimental observations are available to indicate that reduction of platelets in circulating blood below a certain level may lead to haemorrhage and that platelets take part in thromboplastin formation in shed blood. However, the presented experiments indicate that intact platelets are inactive.

Fantl and Hayes (1953) pointed out that platelets are not altered until blood clots, and in dog lymph, which is free of platelets, the initial stages of clotting appear to be the same as in blood (Fantl and Nelson, 1953). Evidence that platelets are not involved in the initial stages of blood coagulation is given

by the fact that the corresponding clotting times, in glass or silicone, of normal and thrombocytopenic blood are practically the same.

The low rate of thrombin formation in horse blood which had an adequate number of platelets and the increase of this rate by added P-lipid (Fig. 1) indicate that the thromboplastic component in platelets was not directly available. This is supported by the observation that the addition to normal human blood of an amount of P-lipid approximately equal to one-quarter of that found in platelets shortened the clotting time markedly (Table 1).

Blood and plasma clotting times in the presence of R.V.V. are not shortened to a degree which would be expected if the platelet factor were freely available. There was a difference between the clotting times of whole blood and platelet-rich plasma in the presence of R.V.V. This is most likely due to the fragility of platelets which even with careful handling suffer a certain degree of damage with consequent release of the active factor.

The addition of P-lipid to either platelet-poor or platelet-rich plasma and freezing and thawing of platelet-rich plasma produce a similar reduction in the R.V.V. clotting time. The disruption of platelet structure which occurs on freezing and thawing liberates a component which now becomes active in shortening plasma clotting times in presence of R.V.V. This effect is near optimal and cannot be appreciably further enhanced by addition of P-lipids.

On the basis of the above results it can be stated that the component of platelets which is involved in the formation of blood thromboplastin is present in masked form and is set free during the coagulation process or by damaging the platelet structure by freezing and thawing. O'Brien (1957, 1958) has reported that mechanical fragmentation of platelets makes available a potent accelerator of the R.V.V. clotting time of plasma.

Although the thromboplastic component of platelets behave like P-lipid, it is not possible to draw any conclusion regarding the type of P-lipid combination.

SUMMARY.

The rate of thrombin formation in diluted horse blood with an adequate number of blood platelets is the same as that of thrombocytopenic human blood.

Clotting time of normal human blood is shortened by the addition of P-lipids isolated from brain.

Russell's viper venom added to human blood or platelet-rich plasma does not produce short clotting times which are expected from the P-lipid content and activity of blood platelets.

Addition of brain P-lipids in amounts corresponding to that present in blood platelets or freezing and thawing of platelet-rich plasma produce equally short clotting times with Russell's viper venom.

Intact platelets are inactive in the initial stages of the clotting process and the component required for thromboplastin formation is masked.

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REFERENCES.

Allen, R. J. L. (1940): Biochem. J., 34, p. 858.
Eagle, H. (1935): J. gen. Physiol., 18, p. 531.
Fantl, P. (1954): Austral. J. exp. Biol., 32, p. 853.
Fantl, P., and Hayes, R. A. (1953): Nature, 172, p. 303.
Fantl, P., and Marr, A. G. (1958): J. Physiol., 142, p. 197.
Fantl, P., and Nelson, J. F. (1953): *Ibid.*, 122, p. 33.
Feissly, R., and Lüdin, H. (1949): Revue d' Hématologie, 4, p. 481.
O'Brien, J. R. (1957): Lancet, i, p. 1213.
O'Brien, J. R. (1958): Nature, 181, p. 420.
Trevan, J. W., and Macfarlane, R. G. (1938): Report of the Medical Research Council for the Year 1936-1937, H.M. Stationery Office, London, p. 143; cited in "Human Blood Coagulation and its Disorders," by Rosemary Biggs and R. G. Macfarlane, Blackwell Scientific Publications, Oxford, 1st ed., p. 308.

A RELATIONSHIP BETWEEN SWINE AND ASIAN STRAINS OF INFLUENZA A VIRUS

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In a recent study of Asian type influenza in Melbourne, it was noted that there was a sharp difference between the clinical attack rate of the under 20 and the over 25 age group, although serological evidence showed that all ages were infected to an equal extent (Keogh, Ferris, Lewis and Stevenson, 1958). These authors attributed the sudden drop in the ratio of clinical to subclinical attacks which occurred in the over 25 age group to prior infection with influenza A viruses prevalent before 1935. This deduction carries the implication that influenza A viruses prior to about 1935 are more closely related antigenically to the Asian virus than are A strains encountered in subsequent years.

The Swine strain of influenza A, which is considered to be related to the 1918 pandemic strain, is the earliest pre-1935 virus available for study. No evidence of antigenic affinity between Asian and Swine strains was found in studies with hyperimmune animal sera (Meyer, Hilleman, Miesse, Crawford and Bankhead, 1957).

This paper presents an examination of the antigenic affinities and differences which exist between the Asian strain, a pre-1935 strain (Swine) and post-1935 strains (PR8 and FM1) employing individual human sera collected during and immediately subsequent to the 1957 Asian influenza epidemic in Melbourne.

Materials and Methods.

Sera. The sera were collected from cases of influenza occurring outside the hospital, from patients admitted with influenza at the time of the epidemic, from blood donors (Red Cross Blood Transfusion Service) and from children admitted to hospital with various illnesses after the epidemic. All were stored at $-20°$ C. before use with the exception of the blood bank sera which were kept at 4° C.

Haemagglutination-inhibition. All sera were treated with periodate to remove non-specific inhibitors before titration. One volume of serum and 1 volume of M/100 KIO_4 were incubated in a water bath at 37° C. for 1 hour; 2 volumes of 50 p.c. glycerol in normal saline were added, giving a serum dilution of 1/4. To 1 volume of each of serial dilutions of sera in normal saline was added 1 volume of saline containing 5 agglutinating doses of

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virus. Racks were shaken and the mixtures allowed to stand at room temperature for half hour before the addition of 1 volume of 1·0 p.c. fowl cells, and readings were made after 1 hour at room temperature. The endpoint was taken as the last tube showing complete inhibition of haemagglutination and the titre expressed as the reciprocal of the initial serum dilution.

Tissue culture neutralization was carried out by the method previously described (Keogh *et al.*, 1958) as a confirmatory check on haemagglutination-inhibition tests.

Viruses. The strains of Swine (200240 (EF)), Asian (PAR 28447), PR8 (EF.28355-6) and FM1 (20242) influenza A were obtained from Dr. E. L. French of the Walter and Eliza Hall Institute. All were egg adapted having been passaged allantoically many times.

The Asian strain used in tissue culture neutralization had been isolated in monkey kidney tissue from a case occurring early in the influenza epidemic. The above Swine, PR8 and FM1 strains were also used in tissue culture neutralization tests after two passages in monkey kidney tissue had produced good cytopathogenic changes.

RESULTS.

Findings given in the following Tables indicate results obtained by the haemagglutination-inhibition method using an initial serum dilution of 1 in 4. However, Tables constructed from results of serum dilutions of 1 in 8 do not differ significantly from the figures presented.

The presence of specific antibody in a 1 in 4 serum dilution was confirmed by the results of tissue culture neutralization tests. Of 435 sera tested for Asian type antibody by both tissue culture neutralization and haemagglutination-inhibition 288 sera were positive in both tests, 97 were negative in both tests, 50 were positive in tissue culture alone and none was positive only in the haemagglutination test. When antibody to Swine, PR8 and FM1 was estimated by the two methods comparable results were obtained.

A total of 553 sera were tested individually for Swine and Asian type antibody. The findings are summarized in Table 1.

TABLE 1.

Correlation of Swine with Asian type antibody in 553 sera, individually tested.

Age	Year of birth	Asian antibody detected		Asian antibody not detected	
		Number of specimens	Percentage with Swine antibody	Number of specimens	Percentage with Swine antibody
0-10	1957-1947	36	36	69	10
11-22	1946-1935	47	31	137	12
23-34	1934-1923	32	81	112	43
35 and over	Before 1923	23	95	97	87

Table 1 indicates that Swine antibody was detected more frequently in sera containing Asian antibody than in sera which lacked Asian antibody. Although the difference appears most obvious in persons under the age of 23 it is actually more or less constant at all ages. This can be shown more clearly by pooling the figures in Table 1. Then 34 p.c. of those under the age of 23 show both types of antibody and 11 p.c. show Swine without Asian—a difference of 23 p.c. In persons over the age of 23 years 87 p.c. show both Asian and Swine antibody and 64 p.c. show Swine without Asian—a difference of 23 p.c.

An examination of paired sera collected from patients during the Asian influenza epidemic confirmed the findings given in Table 1. It was noted that when Asian antibodies rose significantly there was a concurrent rise in antibodies to the Swine strain. These findings are summarized in Table 2.

TABLE 2.

Swine antibody in 103 paired sera from clinical influenza cases (taken during epidemic).

Age	Rising titre to Asian	Percentage with rising titre to Swine	No rise to Asian *	Percentage with rising titre to Swine
11-22	22	40 p.c.	39	8 p.c.
23-34	10	60 p.c.	13	15 p.c.
35 and over	10	90 p.c.	9	33 p.c.

* Asian influenza virus was isolated from 2 cases in this group.

It will be noted from Table 2 that the rising titre of Swine antibodies was more obvious in older persons than in the young. This suggests that the response was of the secondary type in the older age groups and of the primary type in those under the age of 23. This finding was confirmed and extended when sera showing Swine antibody were titred out according to age. Table 3 shows this analysis.

TABLE 3.

Swine titres in sera showing Asian type antibody.

Age	Total positive Swine	Titres			
		4	8	16	32 and over
0-10	25	28 p.c.	40 p.c.	24 p.c.	8 p.c.
11-22	42	31 p.c.	40 p.c.	17 p.c.	12 p.c.
23-34	97	10 p.c.	23 p.c.	22 p.c.	45 p.c.
35 and over	142	3 p.c.	7 p.c.	10 p.c.	80 p.c.

It can be seen that there is a shift to the right with advancing age. Higher titres are found and the response appears to be of the secondary type in persons over the age of 35, who usually possessed Swine antibody prior to Asian infection. Persons under the age of 23 years, with little or no prior Swine antibody, show lower titres in keeping with a primary response. Results in the 23 to 34 age group are intermediate.

A total of 210 sera have been tested for antibody to PR8 and FM1 viruses and tables have been constructed in the same manner as Table 1, correlating results with the presence or absence of Asian type antibody. These results are given in Tables 4 and 5.

TABLE 4.

Correlation of PR8 antibody with Asian antibody in 210 sera.

Age	Year of birth	Asian antibody detected		Asian antibody not detected	
		Number of specimens	Percentage with PR8 antibody	Number of specimens	Percentage with PR8 antibody
0-10	1957-1947	32	53	62	56
11-22	1946-1935	36	80	39	84
23-34	1934-1923	21	100	20	100

TABLE 5.

Correlation of FM1 antibody with Asian antibody in 210 sera.

Age	Year of birth	Asian antibody detected		Asian antibody not detected	
		Number of specimens	Percentage with FM1 antibody	Number of specimens	Percentage with FM1 antibody
0-10	1957-1947	32	78	62	92
11-22	1946-1935	36	94	39	100
23-24	1934-1923	21	100	20	95

In contrast to Table 1 there is no significant correlation for either PR8 or FM1 type antibody with the presence or absence of Asian type antibody.

DISCUSSION.

The studies of Davenport, Hennessy and Francis (1953) on pooled human sera showed that antibody to the Swine strain of influenza A virus was common after the age of 30 years (i.e., in persons born before 1932), but Swine antibody was not demonstrated in younger persons. Isaacs (1957) has recently shown, by testing individual human sera, that Swine antibody was detectable at earlier ages and has postulated the presence of minor antigens common to the Swine and more recent influenza A strains. Our findings based on tests of individual human sera support those of Isaacs in that low titre Swine antibody was detected in children and young adults.

The results shown in Table 1 indicate that infection with the Asian strain of influenza A virus stimulates a concurrent rise in Swine antibody and that this phenomenon is apparent at all ages. The findings in Tables 2 and 3 indicate that this Swine response is of a primary type in the majority of children and young adults, but that in persons over the age of 35 years, most of whom had apparently suffered prior infection with Swine-like strains, the Swine response in Asian infection is of a secondary type. It seems logical to conclude that the Asian influenza virus and the Swine strain share a common antigenic component.

The results in Tables 4 and 5 contrast with those given in Table 1. There is no apparent antigenic relationship between the post-1935 strains (PR8 and FM1) and the Asian virus. In their studies on antigenic composition, measured by antibody absorption, Jensen and Francis (1953) found no Swine component in PR8 and FM1 viruses.

The overall findings in this investigation provide general support for the epidemiological deductions made from this laboratory on Asian influenza (Keogh *et al.*, 1958) in that the Asian virus appears more closely akin to viruses prevalent before about 1935 than to those prevalent after this time. Most of the younger persons found to have Swine antibody at the present time appear to have acquired this antibody as a result of Asian infection, but this can hardly account for Swine antibody detected in persons without Asian antibody. Therefore, we agree with Isaacs that influenza A strains current since 1935 probably share antigenic components with the Swine strain but that the relationship of pre-1957 strains to Swine is not nearly as close as that of Asian to Swine.

SUMMARY.

Evidence is provided that a Swine component is present in the Asian strain of influenza A virus.

The post-1935 influenza A strains (PR8 and FM1) do not appear to be antigenically related to the Asian strain.

These findings are considered relevant to changes in the ratio of clinical to subclinical attacks in the recent Asian influenza epidemic, as noted from this laboratory.

REFERENCES.

Davenport, F. M., Hennessy, A. V., and Francis, T., Jr. (1953): J. exp. Med., 98, p. 641.
Isaacs, A. (1957): J. Hyg., 55, p. 485.
Jensen, K. E., and Francis, T., Jr. (1953): J. exp. Med., 98, p. 619.
Keogh, E. V., Ferris, A. A., Lewis, F. A., and Stevenson, W. J. (1958): In press.
Meyer, H. M., Jr., Hilleman, M. R., Miesse, M. L., Crawford, I. P., and Bankhead, A. A. (1957): Proc. Soc. exp. Biol. Med., 95, p. 609.